Actinomycetes
in
Various Parts
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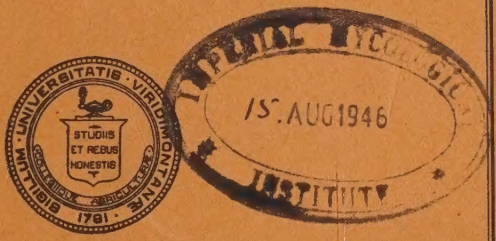
By B. F. Lutman



AGRICULTURAL EXPERIMENT STATION
University of Vermont and State Agricultural College
Burlington, Vermont

APRIL, 1945 — BULLETIN 522

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By B. F. LUTMAN
Plant Pathologist

The presentation of an entirely new viewpoint—one at variance with that generally accepted and supposedly confirmed—is difficult. Every scientist carries in the back of his subconscious mind a series of orthodox images derived from the textbooks which he has studied and the books and papers which he has read. When, however, a science develops too many of these orthodoxies, it is dead; as dead as intercellular spaces in plants are believed to be—although the writer has become convinced that they are alive.

The best way for the author to present this unorthodox view is to review as briefly as possible the steps by which he has arrived at this conclusion, since thus he may hope to either convert others or through their aid discover the turn in the road where he went wrong. Not all of the evidence necessary to sustain his theory is available, but for how many such theories is the evidence complete?

In connection with studies (Lutman, 1941) on the reappearance of potato scab on land which previously had been badly infected but on which, for many years, other crops had been grown and from which the pathogenic organisms seemed to have disappeared, a study of the early infection of very young tubers was undertaken. Skin removed from clean and scabby tubers was mounted in glycerin and examined from above with the microscope.

The potato scab organisms furnish their own dark brown stain and may be readily recognized in the layer between the cell walls of the tissues around and under the lenticels. The twisting and branching filaments of the organisms between the cell walls may be readily photographed, and the extent of the infection as seen from the outside readily followed. The question arises: how far into tubers do these filaments penetrate? Other investigators have tried to follow the organisms into tubers but have been unsuccessful beyond the region of the superficially brown-walled cork and parenchyma tissue.

Most soil actinomycetes are markedly Gram-positive, and by using a modification of Gram's stain, the writer (1941) was able to stain twisting, irregular, branching filaments, which certainly resemble those seen from the surface, throughout the tubers. They could be followed down from the scab lesions between the abnormal, and certainly infected, cells into the starch and vascular tissues of the entire tubers.

A partial confirmation of the ability of the actinomycetes to utilize pectin compounds was found in liquification of a pectin medium (made with gum tragacanth as the colloid, to which the necessary inorganic elements had been added) by a number of brown-pigmented varieties isolated from the soil. On this new medium, growth was better and colonies were larger than on any combination that had previously been tried. It seemed adapted to the requirements of these actinomycetes.

Sections of entirely clean tubers unexpectedly showed intercellular filaments similar to those seen in infected potatoes. At this time, the writer suggested the possibility that, since an actinomyces was known to stimulate the cork cambium of the skin, and especially that under the lenticels, to form scab lesions, the same, or a similar, strain might in like manner stimulate the underground potato stems to swell, store starch, and form tubers. Furthermore, the writer suggested, if soil actinomycetes could stimulate tuber formation in the potato plant, might they not also bear a relation to the formation of other tubers, such as the artichoke, and of sundry fleshy roots?

With this hypothesis in mind, sections were made of various fleshy roots, such as the carrot, beet, turnip, and parsnip, using the modified Gram stain. Intercellular, Gram-positive, irregular lines, similar to the filaments observed in potatoes, could be seen in the cell walls of these roots. The possibility that actinomycetes provide the stimulus for tuber formation or for root enlargement and thickening seemed thus suggested.

Some Pertinent Questions

The potato tuber, however, is only a swollen, colorless underground stem. Why should it be infected, and not the very similar green stems which push into the sunlight and develop leaves? Are the sprouts from the tuber eyes infected with the same organism, so that cells formed in the new plant are also enclosed in a meshwork of filaments? Since flowers are borne on the upper tip of the aerial stems, might not they also be infected? Might the egg cell be free from the filaments and the new plants be clean? Why do seed balls drop so soon? Why do so few mature in the several potato-growing regions? Are they infected in some way by an organism which induces the formation of an abscission layer inside their stems? Is the seed free from the infection? Could one, by growing potato seed in sterilized soil and keeping it free from contamination, produce a sterile plant?

These questions, as well as those of other biologists, the writer tried to answer so far as possible. Slides of young potato tubers were distributed to several interested pathologists and botanists, some of whom examined and commented upon them. Some well-trained observers suggested that the irregular lines to be seen on the slides may not be actinomyces filaments but some other cell organ or part thereof. The question thus raised must be answered first, for if these lines are not fungus fila-

ments, the writer's work would have to do only with an old and, perhaps, solved problem. Accordingly, he has taken up each suggestion made by other workers and has attempted to show its inapplicability. He has also attempted to answer his own questions regarding the nature and distribution of these strands. The objections raised by other workers will be discussed in a later section, but a general presentation of the difficulties of the problem and of the writer's viewpoint will perhaps be of assistance at this time.

THE USE OF DRAWINGS AND PHOTOGRAPHS IN DEPICTING CELLS

The Problem

Drawings and photomicrographs are not in agreement in their presentation of the parenchyma cell walls of tubers, roots, and stems. The drawings ignore certain discrepancies which the photographic plates bring out. In photographs, the so-called walls show the following peculiarities:

1. They are very irregular and twisting.
2. They appear as one, two, or three walls, often at different focuses.
3. Parts of the walls may take an intense stain, while other parts may be lightly stained or missing.
4. A wall may split, with part of it seeming to enter the body of the cell.

At the corners of the cells, all these conditions are especially confusing. In drawings, all these irregularities are ignored. The walls are drawn as continuous and single, they are made to meet at the cell angles (except for an intercellular space), and none of them protrude into the cell lumen.

Any series of drawings and photographs of thin-walled, parenchyma tissue will show the points referred to above, but two papers by Artschwager (1918[1], 1924) may be cited since they are generally available and since they present unusually clear photographs and well-executed drawings. For another example, also easily obtained, which shows that these characteristics of photographs are not confined to potato tuber cells, the reader is referred to the photomicrographs in Sinnott and Bloch's paper (1941) on division in vacuolate cells of a number of plants.

Difficulties in Interpretation

The plant cells considered in this bulletin are those of the thin-walled type only, and many of the comparisons will be made between walls as they are photographed and as they are usually drawn.

In the back of every botanist's mind are definite patterns for the walls of parenchyma cells, and the influence of this extends from freshman

[1] Jour. Agr. Res. 14:221-252.

notebooks to doctors' theses. The evidence on the structure of thin cell walls of plants offered by an easily-obtained photograph is simply ignored. In a drawing, the walls are corrected to conform to those of a geometrical figure with curved sides. Intercellular spaces never appear except at cell angles.

The time has come to take a look at some of the discrepancies and to determine which is correct in the reproduction of parenchyma walls—the photograph or the drawing. A number of these discrepancies will be taken up in detail later. At the present time, only two propositions need be considered fundamental:

1. Thin walls of mature cells are never plane surfaces.
2. Plasmolysis and "poor fixation" are not so common as is generally believed, and many plant anatomists do not recognize "poor fixation" when they see it.

The fundamental basis for much of the present error in interpretation lies in the facts that thin cell walls are never plane but are always curved surfaces and that plant anatomy is studied almost exclusively by the examination of thin mounted sections under the microscope. Sections of cell walls, whether reproduced as photographs or as drawings, can show only two dimensions when put on the film or drawing paper, length and breadth. The third dimension is not drawn because its presentation is inconvenient and would require an accomplished artist. In a photograph, sections of the third dimension do show, but they are partly out of focus and have usually been misinterpreted.

The complex curved shape of plant and animal cell walls (the latter are also usually thin) may be seen as they really are in the artistic and accurate drawings of elder pith and precartilage cells made by F. Schuyler Matthews from models built up from sections under Dr. Lewis' direction (Lewis, 1923, 1933). While the edges of these precartilage cells are curved lines, they are not wavy or twisting, and there are neither "knots" nor sharp "kinks" in them such as frequently show in photographs of many plant parenchyma cells. These appearances will be discussed later. Marvin (1939, 1944) has found similar complicated geometrical figures and has found also that the pith cells of many plants cannot be readily separated and that in the case of *Eupatorium perfoliatum* they can be separated only in the internodes below the growing regions.

It does not seem to have occurred to cell morphologists to represent the three-dimensional appearance of cells as seen in optical planes. At any rate, they have avoided making such representation.

A typical cell, about 70 microns in diameter, of a young potato tuber is shown in the top drawing of Fig. 1. The wall of this cell and parts of the walls of surrounding cells are shown only in cross section. In other words, the representation is only two dimensional. The intercellular spaces at the cell angles are left clear.

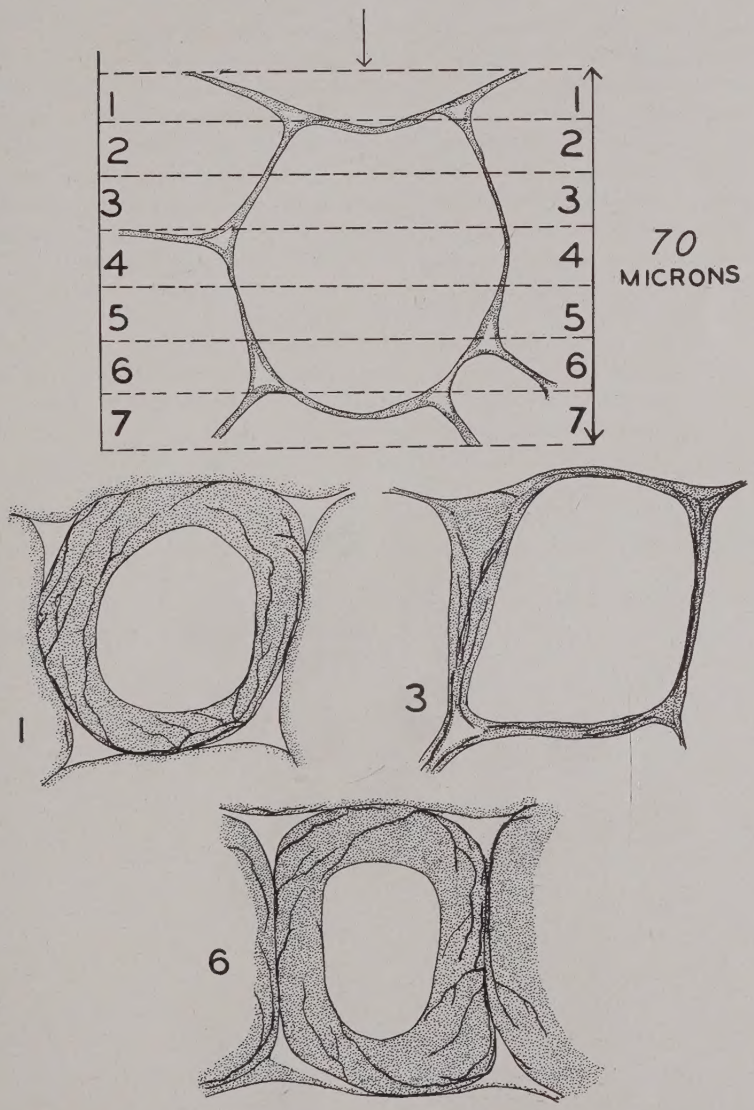


Fig. 1. Cell in side view (top) and a series of sections cut from it, as seen from above, to illustrate the appearances which the cell would have at various levels. The bowed walls and the intercellular spaces show markedly in sections 1 and 6; less in section 3. Diagrammatic.

But, suppose this cell had been sectioned at 10-micron intervals in a plane at right angles to the plane of the drawing. The various sections

would have been removed at the levels indicated by the numbers 1 to 7 in the upper figure. If such a series of sections were examined from above, in the direction of the arrow, sections 1, 2, 6, and 7 would be different from sections 3, 4, and 5, in that the former would show part of the walls of the cells above and below, and section 3 would differ from the others in showing no intercellular spaces. To illustrate, section 1 would show a central unshaded portion, the cell lumen; stained areas, indicating the broad-appearing, sloping walls; and clear spaces at the corners, indicating the large intercellular spaces. While intercellular spaces have been largely ignored by most anatomists, in some tissues they occupy almost as much space as do the cells themselves. The lower drawings of Fig. 1 show the sloping walls as seen in sections 1, 3, and 6.

The irregular, inner border, stained intensely, might suggest to the observer that it is the result of bad fixation and shrinkage. If, however, the illumination were decreased, the sloping walls at the sides of the cells might be seen and the real margins where the walls were cut might be located as a granular line of demarcation. About one section in three would show marked examples of "poor fixation" and "shrinkage," although every section would show a little on one or more sides. Section 3 misses the slanting concave walls, and the walls, therefore, appear relatively narrow, as reproduced in the drawing.

The cell wall in the plane of the sections can be found only rarely and usually then is incomplete. A few such walls, however, are shown in some of the drawings and photographs in this bulletin.

In Fig. 35, showing turnip cells, the true walls are not shown nor do they show in any cells stained by the usual technique, for the twisting outlines of the cells are not the true walls but some form of an intercellular inclusion. In the ordinary slide-making technique, where the application of an anilin aqueous stain is followed by dehydration in alcohol, only the coarser inclusions retain the stain and many of the finer branches are decolorized. Some of these finer branches may be seen, still stained, in the turnip cells to which reference has just been made. These are not folds in the wall nor the crumpled margins of the shrivelled cytoplasm. These matters will be discussed in detail later. At this point, only this different viewpoint regarding cells is presented, in order that the author's theory of intercellular inclusions may be understood.

THE ACTINOMYCES INTERCELLULAR INCLUSION THEORY

The actinomyces intercellular inclusion theory which the author has formulated on the basis of the work described in this bulletin may be stated briefly as follows:

> The material between plant cell walls is composed of a variety of pectins and gums, cell exudate, and some protein. The intercellular spaces are large in mature tissue, but the cell walls facing these spaces are covered with this heterogeneous mixture. When

the usual basic stains are used, this material stains faintly, while the portions now to be discussed stain more heavily.

Actinomyces filaments are imbedded in this mixture, in some plants running through clear tubes which they have apparently formed by dissolving the mixture. The filaments branch, become more delicate, and fray out; and their general direction is that in which growth had occurred in the tissue. They are especially concentrated, or at least are most easily observed, at the corners or angles of the cells. The cellulose walls of the cells are very thin and are usually unstained.

In a series of sections of a cell, the filaments appear in part in one section but are cut off and reappear in the next section. Some of them spread over the bulging and sloping sides of the cell before they are cut off (Fig. 1).

The actinomyces theory is in direct conflict with the generally accepted middle lamella theory of Mangin.

The evidence which will be offered in behalf of the writer's contention is almost entirely morphological and microchemical. He is well aware that the most convincing proof of the fungus nature of the filaments would be to isolate an organism from the tubers or roots into pure cultures. In spite of repeated trials, however, complete success in this respect has not been attained, although some progress has been made by using pieces of potato tuber and the pectin medium already mentioned.

Growth of some organism, apparently an actinomyces, was observed on the pieces of potato tissue, and examination under the microscope confirmed the observation. Transfers to the same pectin medium were unsuccessful, the potato tissue apparently being necessary for growth. Moreover, the pieces of potato could not be depended upon for actinomyces colonies; sometimes they would appear, but on some pieces or in some trials none could be found. It is difficult to explain this erratic behavior, in view of the fact that the infection of the tubers by actinomyces filaments seemed to be general under the microscope. The pectin medium would seem to lack some factor essential to the nutrition of the fungus, or there may have been some other factor preventing growth except at certain times. The symbiotic relation between the potato cells and these inclusions must be a very close one, and apparently the environment and food must be exactly right to entice the latter to an independent mode of life.

The isolation of actinomyces cultures from living potato tubers is not to be confused with the Schanderl-Rippel controversy over the fixation of nitrogen by plants that are not legumes through the activities of organisms present in them. The one paper seen on this subject, that of

Rippel (1941), indicated that these organisms isolated by Schanderl, but disputed by Rippel, were rod-shaped.

The attempts to demonstrate that the strands are actinomyces filaments and not cell walls or middle lamellae have been based on the examination of sections of tissue under the microscope, aided by a study of the size, behavior, staining, and microchemical reactions of these inclusions. Much of the demonstration has been done by photomicrographs. In examining these photographs, cytologists should remember that cell walls and the so-called middle lamellae would be plates and, however they might be cut, would show at every focus continuous lines which would unite with the other walls or lamellae at the cell corners. Cell walls can never be triple nor can they twist over each other. In sections, they should not appear as twisting lines or lines with sharp horseshoe-shaped bends.

REVIEW OF LITERATURE

Work on the Composition of the Intercellular Region

The statement made a hundred years ago that very little is known of the composition or structure of the material between the cells still holds today. To the German botanists, this material was simply the "Zwischenstoffe," the filling-material between the cells.

The modern viewpoint is largely due to the investigations of Mangin. Mangin published his results in a series of short contributions between 1888 and 1893, but his elaborate memoir, a series of articles (1891-1893), was well illustrated and should be consulted to get any comprehensive view of his work.

The new stain, ruthenium red, which helped him to advance our knowledge of the intercellular region was discovered by Joly. It has a very selective staining action on pectin and pectin compounds, leaving other cell organs uncolored. It can be used in a very dilute aqueous solution, 1-5,000, and after being stained, sections can be made fairly permanent without loss of brilliancy by placing them in glycerin. Freehand sections were used exclusively by Mangin.

By the use of this dye and of various microchemical tests, Mangin arrived at the conclusion that the material between the cell walls was not homogenous, but that portions of it, which stained a darker red, were the true middle lamellae. Mangin believed his middle lamella to be pure pectin, probably a calcium pectate. On either side of it was a mixture of pectin and cellulose and, finally, the cellulose walls.

Because of their importance, a committee of chemists has determined the nomenclature of the protopectins, pectins, and pectic acids which have been isolated (Report of Pectin Committee, 1944). However, this has not helped the biologist as yet in his attempts to locate and differentiate them inside the plants. Bonner (1936) closes a general review of

their chemistry and physiology with the statement that " . . . . for a complete understanding of pectic substances, much remains to be done, by both chemist and botanist." It is not necessary to review the microchemical tests by which Mangin attempted to support his tinctorial results. They are summarized by Harlow (1927).

Still another factor was introduced into the question of the composition of the middle lamella by Ritter (1925), who found that 75 percent of the lignin of wood is located in the intercellular region while only 25 percent is in the cell walls. The middle lamella was defined by Ritter as "the isotropic peripheral layer of the cell wall, including the irregular masses of isotropic material commonly formed when three or more cells join."

This definition of the middle lamella, especially as it occurs in the secondary, thickened wood cells, takes the question back almost to the old "middle-material" of a hundred years ago. The same results and the same broad definition of the middle lamella appear in the discussion of Kerr and Bailey (1934), who find it "desirable to restrict the term to the truly isotropic *intercellular* substance which separates the primary walls of adjoining cells." These authors were investigating the middle lamellae in wood tissues where the cell walls had been thickened by secondary deposits.

The work of Mangin had been preceded by some investigations by Wiesner (1886) and his student, Krasser (1886), both of whom made claims that the plant cell was surrounded by a layer of living material, protein in composition, which played an important role in the intake of food and the excretion of waste materials. Between this outer living dermatoplasm and the inner living cytoplasm was interposed the thin cellulose wall, which was non-living. The two living layers must have communicated in some manner through this cellulose barrier. Wiesner's work has been ignored in all recent discussions of the intercellular region.

The protein nature of the middle lamella has been more recently asserted by Dauphiné (1934) based on a series of tests made on fresh, untreated plant sections. After their immersion in a solution of sodium hypochlorite (which dissolves all proteins), he arrived at the conclusion that the middle lamella is not pectin nor a calcium pectate, but a protein. The protein reactions of the intercellular area to staining are more brilliant than those of the cell cytoplasm.

The cytology of the formation of the middle lamella and of the cell plate in higher plants after nuclear division has not received much attention in recent years. The work of Timberlake (1900) and Allen (1901) seems to have been generally accepted, except in some special cases. According to Timberlake's explanation, the cell plate is formed by the fusion of a row of granules along the equator of the spindle. Preceding the formation of this row, however, a clear zone shows in the spindle, the contents of which stain orange in the triple stain, indicating cellulose or

other related carbohydrates. After the granules have fused, the row splits to form the cell plate, no filaments or fibrils being noted. In the split the new cell wall is deposited. These cell plate studies seem to have been made from the side view. It may well be that a survey from the end view might alter the interpretations, and that the use of stains other than the very much over-worked triple or hematoxylin stain might be worth while.

Work on the Presence of Fungi and Actinomycetes in the Intercellular Region

In general, fungi and bacteria use the intercellular region as the easiest path to the interior of a plant root, tuber, or stem. The mixture of pectins, proteins, and gums is apparently more readily dissolved than the cellulose walls which must otherwise be penetrated. The presence of large intercellular spaces with a water-saturated atmosphere and some available oxygen may also cause them to choose intercellular routes.

Some of these organisms, such as the soft rot bacteria, dissolve all of the middle material, and the cells, left without their cementing layer, fall apart. The majority of the fungi, such as the rusts, smuts, and imperfecti, dissolve only a path for themselves and leave the cementing layer almost intact. The literature on these intercellular parasites would include at least half of plant pathology.

Various other soil organisms, such as fusaria and the mycorrhizae, are also to be found in the superficial layers of the roots of higher plants. Some of these organisms are saprophytic and others slightly parasitic, while some are so pronouncedly parasitic that they destroy not only cells and tissues but the entire host plant. Bernard (1902) found them to be the general stimulus to growth and tuberization in such plants as the orchids, but in the potato he could not obtain any effects from the fungi which he isolated. His work has been continued by Magrou (1921), but the evidence still seems to be insufficient. The potato still keeps its secret of tuberization. However, the observations of Parmentier, who popularized this plant in France, are interesting: when potatoes were grown in some new fields into which they had been introduced, the plants flowered and produced seed in abundance, but formed no tubers.

Attention might be called to the theory of Constantin (1922) regarding the potato in its relation to fungus infections. He suggests that the supposed ancestor of the potato, *Solanum maglia,* was thus infected, but that the potato itself, when carried to cooler climates, lost the fungus which it had in the tropics; that fungus and cold act together to induce tuberization in temperate climates, but that either factor makes tuber formation possible.

A review of the extensive literature on tuberization, including a very complete summary of the older literature and of later observations, together with the theories involved, appears in Rayner's monograph (1926,

1927), and its biological significance in Burges' discussion (1936). The symbiotic relationship of the mycorrhizal fungi and the enclosing higher plants is borne out in the recent investigation of MacDougal and Dufrenoy (1944).

The mycorrhizae which have been most thoroughly studied occur on the roots of forest trees, especially pines. They are very definitely fungi which invade the root and its cells as tangled masses. Plant parts other than the roots may be invaded, even the ovaries, as in the orchids. Only a few examples of these invading fungi have ever been held to be actinomycetes.

The actinomyces group, however, has received very little attention and the only investigations which attribute the invading organisms to actinomycetes are those of Beijerinck (1900), Arzberger (1910), Peklo (1910), Dufrenoy (1920), and Person and Martin (1940). Beijerinck found a variety of plants whose roots were infected superficially by actinomycetes. Arzberger ascribed the tubercles on *Myrica cerifera* to a species of actinomyces, confirming work that had been done on other species of *Myrica*. In fact, Peklo claimed to have isolated an actinomyces from another species of *Myrica*. He found that the actinomyces filaments are very coarse (1.4 to 4μ) and penetrate the cell walls of a very limited region of the growing top of the young tubercle. The cells themselves are penetrated and some of them become filled with the growth, which stimulates the formation of much-branched tubercles.

Peklo's observations, while somewhat unusual, are worthy of inclusion, since he presents the actinomyces problem from both plant and animal standpoints. His work, however, has not been generally accepted and needs confirmation.

Dufrenoy found an actinomyces-like organism in the roots of *Adenostyles albifrons* in the Pyrenees Mountains in France. The organism was believed to penetrate the cells. Person and Martin found an actinomyces in sweet potato tubers, where it caused a soft rot.

The occurrence of actinomycetes in potato scab is well known, but the description by the writer (1941) of its spread throughout the tuber has not been received with favor by plant pathologists (Heald, 1944, p. 314). The confirmation of the presence of actinomyces filaments between the cork and sub-cork cells by fluorescent microscopy by Richards (1943) should receive attention. The fluorescent technique is valuable in identifying and separating tuberculosis and other acid-fast organisms (to which the scab actinomycetes are related) from others not having this acid-fast characteristic. Through the use of this technique, fluorescent filaments were found in potatoes, where the writer had described them after using a different staining technique.

STAINING PROCEDURES AND MICROCHEMICAL TESTS USED IN THE STUDY

The object of the staining procedures and microchemical tests used in this study, none of which is specific but which are useful since they support one another, is to ascertain two properties of the filaments (or lamellae) included in the walls: (1) their difference from the other cell organs; and (2) their protein or non-protein composition.

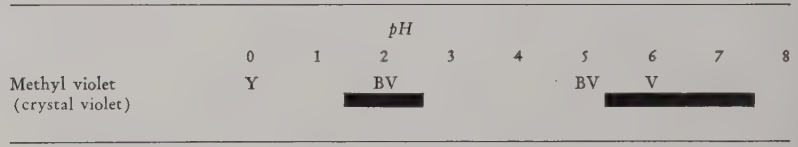
From tests made in this study and work of other investigators, the following conclusions may seem to be fairly well established: (1) these filamentous inclusions in the walls are not of the same composition as the cell, since they have a pH of about 5.0 to 5.2 as compared with a pH of 6.0 in the cell; (2) these filaments are composed of a very dense protein material which reacts in the same way as protein to the tests generally used.

In this discussion, these intercellular inclusions have been named filaments, but Mangin saw them as plates and many plant pathologists and botanists to whom the writer submitted slides for examination were apparently unable to decide as to their morphology. The problem is really a very puzzling one and involves the third dimension in microscopy. The next section will be devoted to demonstrating, as well as is possible, the filamentous nature of these inclusions.

Stains and Staining Methods

1. *Modified Gram Stain.* When the staining time was lengthened from minutes to hours, the crystal violet-iodine combination was found to be retained by the filaments under potato scab lesions (Hutchins and Lutman, 1941). These filaments appeared sharply differentiated from the potato tissue because of their dark-blue color, as contrasted with the red-violet color of the latter.

A drop of the crystal violet stain turns dark blue as it mixes with a pH 5.2 buffer solution, and it turns red-violet as it mixes with a pH 6.2 buffer solution. No investigations seem to have been made on these dyes until a number of them were tested in the research laboratory of the Eastman Kodak Company, to which institution the author is indebted for the following data:



Methyl violet has two ranges as an indicator. It varies from yellow (Y) at pH 0 to blue-violet (BV) at pH 2, and from blue-violet (BV) at pH 5 to violet (V) at pH 6. The second range is the one in which the crystal violet and Gram stains act.

The crystal violet and Gram staining reaction of plant tissues lies in the second range of color changes. This reaction seems to have entirely escaped the attention of cell technologists.

The reaction of potato tubers, which has been thoroughly investigated, has been found to lie slightly below or above pH 6.0. Small (1929, p. 268) states that parenchyma tissue had a pH of 6.2, but that there was little doubt that the internal pH was nearer 5.9 and the wall was in the range of pH 5.9, although the cell sap was pH 5.2-5.9. This places the tuber cell walls in the upper part (the violet end) of this color range.

The actinomyces mycelium is too fine to permit making pH determinations on it, although the writer has tried to do so. However, organisms of this type will grow on a medium having a pH of 5.4, and potatoes will scab in fields with a pH of 5.4. In order to prevent scabbing, the soil reaction must be reduced below a pH of 5.0. The reaction of the mycelium of some of the larger fungi, such as *Rhizopus nigricans,* was determined by Robbins (1924) to be about pH 5.0. These reactions place the actinomycetes in the lower part (the blue-violet end) of this second color range, probably between pH 5.0 and 5.2.

The color difference between the inclusions and the tissues is more marked in the potato than in the other plants studied, although Dufrenoy (1920) noted the same colors in sections of *Adenostyles albifrons* infected with a supposed actinomyces. He states, "It is best to stain with carbolic crystal-violet, than to differentiate with amyl-alcohol, which stains the endotroph a deep blue, while the host tissues display a light metachromatic rose-colour."

The gentian violet-iodine stain is an excellent one for nuclei and for lignified or suberized walls, as was pointed out by Zimmermann (1893) when he used a Gram's stain for this purpose. His technique differed from that generally used in that after holding the specimen one minute to a few minutes in a 3 percent anilin-gentian violet solution and immersing it briefly in the iodine solution, he differentiated in alcohol and cleared in clove oil, although xylol could be used. Zimmermann's technique does not seem to have been widely used but recent modifications of the triple stain with an intensification of the color by treatment with iodine resemble the Zimmermann method and are in high repute with many workers, since they define the chromosomes sharply.

The staining methods used for potato tuber sections by Hutchins and Lutman (1941) are not applicable to the other organs of the potato. Potato tuber cells are usually filled with starch grains which seem to hold the gentian violet-iodine combination so tenaciously that a half to a full hour of decolorization in absolute alcohol is necessary to differentiate the filaments from the enclosing walls. Coarser filaments in tuber cell walls delay decolorization. The process must be stopped at

a point where the finer filaments retain the stain, whereas a few minutes more exposure would remove it entirely from the finer filaments.

Sections of stems, even after a 24-hour immersion in 5 percent crystal violet and Gram's iodine solutions, require only a fraction of a minute in the alcohol (just enough to insure dehydration) to remove the stain from everything except the actinomyces filaments (Wheeler and Lutman, 1942). Leaf sections, however, are more difficult to differentiate, for even the brief exposure in absolute alcohol necessary to dehydrate their tissues will sometimes bleach them, so that the alcohol has to be poured on and off as rapidly as possible, and differentiation has to be continued in xylol.

The retention of the crystal violet stain by the filaments seems to be physical rather than chemical. The modified Gram's stain, on the other hand, makes use of the fact that the actinomycetes are usually Gram-positive, supposedly a chemical reaction. The best staining results, however, were obtained with this latter stain, since even the very fine filaments, which are only a fraction of a micron in diameter, could be followed because their dark-blue color contrasted with the reddish-purple hue of the other cell organs. The stain fades on long keeping in xylol-Canada balsam, so that observations on the finest branches must be carried out within a week or two after the preparation has been made. In fact, the best method of decolorization is to carry the slide into alcohol and then to allow it to remain for some hours in xylol. However, too long an exposure to Canada balsam in xylol will bleach the thinner filaments. This Hutchins-Lutman modification of the Gram stain is superior, therefore, if the slide is examined at just the right time; but it has one fault, the blue filaments do not photograph as brilliantly as if they were red or black.

Another annoying occurrence is the formation of fine globules (apparently of the anilin oil) in the crystal violet solution. Even repeated filtering through a hard filter paper, and frequent changing of the iodine solution and of the alcohol used in differentiation does not result in successful slides. In fact, this globule deposit is at its worst just after a new lot of the anilin-crystal violet is put into the staining dishes. After 30-40 slides have come through spoiled, the globules begin to disappear. So, as was suggested in the paper by Wheeler and Lutman, the best procedure is to allow the anilin-crystal violet solution to stand for a few days, and then filter it. If necessary, repeat this procedure. The superfluous anilin oil seems to settle out and become a deposit on the sides of the staining jar.

2. *Brilliant Cresyl Blue.* Brilliant cresyl blue stain is used in a 1 percent aqueous solution on either fresh or fixed material. No differentiation is necessary other than the use of absolute alcohol for dehydration before the sections are placed in clove oil.

Brilliant cresyl blue is really a violet and seems to have the same

hydrogen-ion colorimetric reactions as crystal violet on potato tuber sections. This stain has not been investigated by the Eastman Kodak Company research laboratory, but when the stain was added to buffer solutions having a pH 5.0 to 6.5, dark blue and red-violet appeared as with crystal violet.

3. *Derrien and Turchini's Stain* (1924). Dauphiné (1934) recommends the Derrien and Turchini stain as one of the best tests for protein and trial of it in the present work has justified this praise. The stain was used by its originators on chick embryos to differentiate proteins from fats, and also on various plant tissues, such as sections of germinating seeds and on aleurone grains. It is inexpensive, simple, and certain, as well as permanent. Its grays and blacks are easy to photograph. The principle is the same as that of the old iron-tannic acid ink. Since it may not be generally known, the technique used in this work is described:

1. The sections on slides in an aqueous solution are mordanted for 15 minutes in a 10 percent solution of tannin in 30 percent acetic acid;
2. Washed in distilled water, which should be changed after every two or three slides;
3. Immersed for a few minutes in a 1 percent solution of ferric chloride; and
4. Washed in water, dehydrated, and mounted in balsam.

This procedure stains the intercellular filaments an intense black, and the nucleus and cytoplasm a dark gray.

This stain, as its originators explain, is a modification of Zacharias' test for proteins, in which Prussian blue is deposited on the protein, although the use of tannin is credited to Overton and Poulsen. The use of tannin and iron was revived by Salazar in 1920, improved (1944), and used on many types of animal tissues. Sharman (1943) used tannic acid and iron alum on sections of shoot tips of *Agropyrum repens* to stain the cell walls, but the writer's observations indicate the stain was really not located in the cell walls, but in the intercellular inclusions among which were the strands of actinomycetes.

4. *Bernard and Magrou's Stain.* Bernard (1902) and, later, Magrou (1921) used a stain in studying the mycorrhizae of orchids and the potato which is so different from any other stain in use that it was thought worth a trial. Fixed and sectioned material, from which the paraffin has been dissolved and which is placed in absolute methyl alcohol, is transferred to a stain made up of eosinate of methylene blue, 7 grams; eosinate of toluidine blue, 1.5 grams; toluidine blue, 0.5 gram; and absolute methyl alcohol, 490.0 cc. One cc. of this mixture is placed on the sections for one minute. The slide is then washed off with 4 cc. of distilled water, neutralized with neutral red. After 10 to 15 minutes, the neutral red solution is washed off with tap water. The sections are now

differentiated with a solution of tannin orange to a light pink, dehydrated with absolute methyl alcohol, cleared with toluene, and mounted in cedar oil.

The writer believed that eosinate of methylene blue was the same as Wright's blood stain, but he was later advised that Jenner's blood stain is more nearly a true methylene blue eosinate. The "distilled water neutralized with neutral red" was interpreted as a weak (rose) solution of neutral red. The "tannin orange" is the same type of stain as Orange G but Orange III was believed to be a little nearer and was used in a 0.5 percent solution. No account was given in the original paper of the results to be expected on the various cell organs, but the actinomyces filaments in the wall stained an intense blue.

5. *Wright's, Jenner's, and Giemsa's Blood Stains.* The use of the eosinate of methylene blue in the Bernard-Magrou stain suggested a trial of Wright's, Jenner's, and Giemsa's stains, which are often used on human blood to differentiate parasites and the various types of leucocytes. Considerably longer staining times were used than those recommended for work with blood. The results obtained were all about the same; the intercellular inclusions were stained a dark blue.

6. *Other Stains.* Observations on the retention of other stains by the hyphae of the intercellular actinomyces have led the author to the conclusion that prolonged application of several other dyes, followed by decolorization will afford essentially the same results. For example, a 12-hour staining in a strong aqueous safranin solution, followed by a differentiation in alcohol, leaves the filaments red. A similar staining in iron alum-hematoxylin with a bleaching in an iron-alum solution, leaves the filaments gray-black. Molisch (1923) recommends a number of basic stains for the middle lamella, including safranin, methylene blue, acid green, and neutral red.

Microchemical Tests

Microchemical tests were made for pectin and for protein.

Pectin Tests

Attention should be called at once to Molisch's statement (1923): "Unfortunately at the present time no specific test for pectin is known."

Ruthenium Red. A very dilute solution (1-5,000) of ruthenium red dye gave positive results in all trials on sections of potato, artichoke, beet, etc., indicating the presence of pectin or pectin-like slimes, i.e., soluble pectin, or, possibly, such insoluble pectin compounds as calcium pectate. Conrad (1926) found insoluble pectins in potato tubers (an actinomyces might not be able to use them unless provided with the proper enzymes for rendering them soluble). No recent investigator puts too much reliance on these stain tests for pectin. The two tints of red which are obtained by this weak dye are a light-rose color for the

ground substance of the intercellular materials and a dark red for the irregular inclusions, plates, or filaments. Mangin (1893) has shown the difference very clearly in his figures. Actinomyces filaments are very fine, and the color may be lost from them if the sections are mounted in weak glycerine.

Smears from cultures of soil actinomycetes, fixed by heat, stain readily in the usual ruthenium red aqueous dilutions, showing further the lack of specificity of this dye for pectin.

Protein Tests

Millon's reagent and nitric acid were used on microtome sections of fixed and imbedded potato and artichoke tubers, as well as on hand sections of fresh tubers.

Millon's Reagent. The reaction with Millon's reagent was not brilliant, but the dull red color was as pronounced for the actinomyces filaments as for the nucleus of the host plant and was located distinctly within the filaments and not in their enclosing walls. In other words, the protein was located within the tube. This is important, since the enclosing walls of the filament might be the plasma membranes of the two potato cells adjoining it, which consist of protein, and would thus give a positive Millon reaction.

Nitric Acid. After cold nitric acid was placed on the sections, they were gently warmed for a few minutes and then allowed to stand for an hour. The yellow, xanthoproteic reaction appeared in the same regions of the filaments as did the dull red color of Millon's reagent. Better results were secured when freehand sections of fresh Jerusalem artichoke tubers were thus treated with nitric acid than when potato sections were used, owing to the absence of starch grains inside the artichoke cells. Under the microscope, the yellowed actinomyces filaments appear to form a framework, as of a house without roof, walls, or floors. They branch and fuse at the corners in a complicated pattern, but no plane surfaces appear, only the yellow, branching strands of the actinomyces in the intercellular spaces.

The Biuret Test. No test for proteins is very satisfactory, but the biuret test and the Derrien and Turchini stain were regarded as the best by Dauphiné (1934). Protein generally gives a violet color (similar to that of biuret, a wine) when treated with a copper sulphate solution and sodium or potassium hydroxide, although the result may appear negative if only traces of protein are present.

Sections of fixed material of a number of plants, including potatoes, artichokes, carrots, and beets, gave a distinct biuret color, confirming the observations of Dauphiné.

Zacharias' Test. The Derrien and Turchini stain is an improvement on the Zacharias test, which is one of the early tests for protein. Molisch

(1923) regarded this test as useful when confirmed by other chemical reactions.

1. Slides are placed for 30-40 minutes in a solution composed of one volume of a 10 percent aqueous solution of potassium ferrocyanide and one volume of glacial acetic acid. This mixture must be used fresh and frequently renewed.
2. The slides are washed briefly in 60 percent alcohol. This is best done by flooding the slides with the alcohol.
3. The slides are transferred to a 1 percent aqueous solution of iron chloride. Proteins take on a light-blue color from the formation of iron ferrocyanide (Prussian blue).
4. The slides may be washed in water, dehydrated, and mounted in balsam. The color is not readily soluble and the slides may be kept and examined for some days. They gradually fade, however, the Prussian blue apparently being largely of the soluble rather than the permanent type.

The iron-ferrocyanide test on sections of potatoes and artichokes, beets, carrots, etc., was markedly positive for the filaments enclosed between the cell walls.

THE ACTINOMYCES THEORY COMPARED WITH MANGIN'S THEORY

Since the term "middle lamella" has come to have many different interpretations, the writer is using Mangin's definition as given on page 337 of the summary of the final paper of his monograph (1893).[2] Mangin's methods were carefully tested personally by Molisch (1923) and his results accepted, freehand sections and the tests suggested by Mangin being used.

To clarify the Mangin theory in its relation to the present observations, it should be stated that the ordinary botanical textbook figures, by which Mangin is known to most botanists who have not read his papers, are misleading in that they show only the simplest middle lamellae, and not the more complicated lamellae typical of Mangin. Plate I attempts to present Mangin's very complicated observations in the form of colored diagrams, and Fig. 4 is redrawn from Mangin.

Fig. A of Plate I, a typical textbook presentation, shows the middle lamellae as homogenous lines between thick secondary walls. This draw-

[2] "L'acide pectique se recontre principalement à l'état de pectates insolubles, le plus souvent à l'état de pectates de chaux; dans tous les tissues mous, adultes, où il occupe la région externe. Là, il forme: (1) la lamella moyenne ou substance intercellulaire, et sert alors de ciment entre les diverses cellules des tissus; (2) à la surface des espaces intercellulaires, il forme un revètement très mince dans lequel se trouvent englobés les cristaux d'oxalate de chaux que présente souvent la membrane; (3) le longe de la ligne qui limite la surface d'adhérence de deux cellules voisines, les pectates insolubles forment une bordure assez épaisse souvent saillente dans espaces intercellulaires, et constitue ainsi des cadres limitant la surface d'union des cellules; (4) sur les bordes de ces cadres et souvent mème sur la surface des revètements intercellulaires, il se produit des excroissances affectant la forme de boutons ou de bâtonnets simples ou ramifiés, constitués exclusivement par des pectates insolubles; parfois mème les pectates remplissent les espaces intercellulaires."

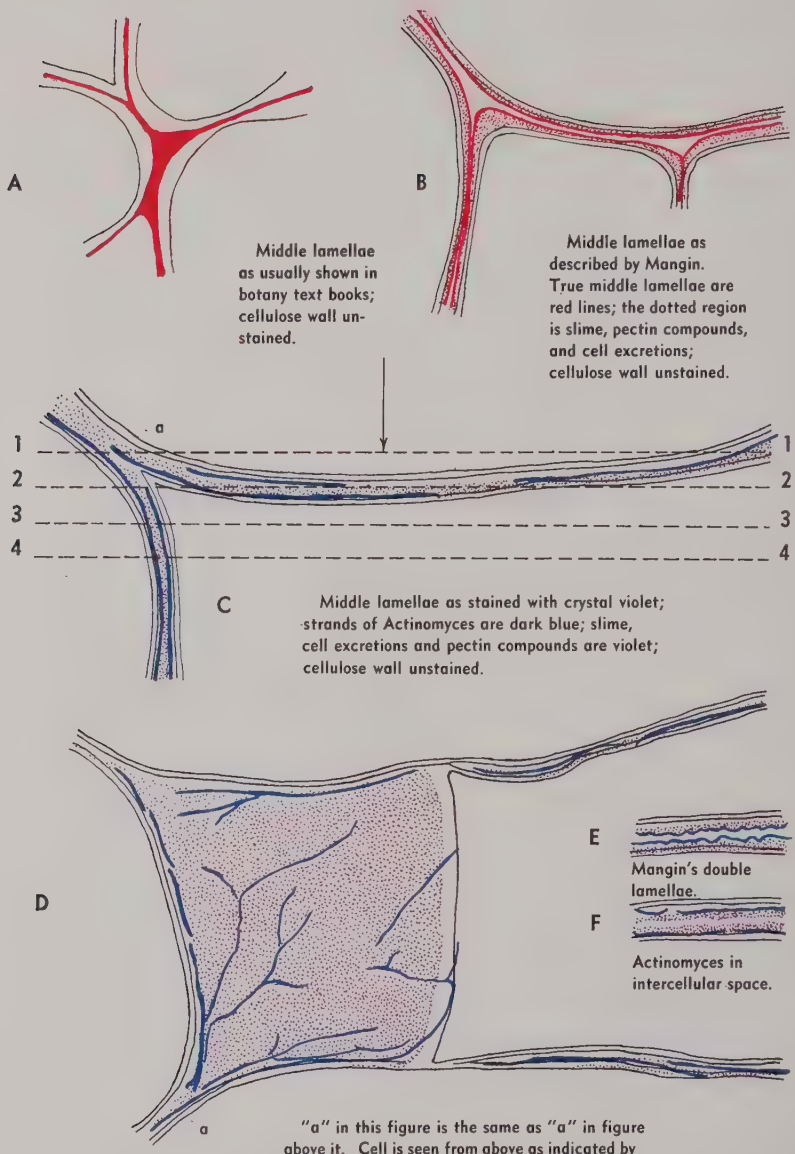


PLATE I

Mangin's middle lamellae compared with the actinomyces filaments in the intercellular layer. The colors approximate those of ruthenium red or crystal violet in its two shades. These cells are based on sections of potato or artichoke tubers but might represent the thin-walled cells of any plant. Diagrammatic.

ing is incorrect in that only a single homogenous inclusion is shown, rather than a light-stained area and a dark-stained area. Fig. B, derived partly from Mangin's figures and partly from his descriptions, is also in textbook usage. Dark-red plates are imbedded in a matrix of lighter-staining slime. The double plates usually become single at the ends or sides of a cell but are double at the cell angles.

The ruthenium red stain which Mangin used for comparison gives only the two shades of red just mentioned. The writer has carefully tested this stain using water mounts after applying it and examining the sections immediately.

For comparison with it in water mounts, the best results have been obtained with a 0.5 percent aqueous solution of brilliant cresyl blue, which stains a dark blue in some parts and a light violet in other parts.

Paraffin sections of fixed potato or artichoke tubers about 10 microns in thickness were stained for 15 to 30 minutes and were then washed in tap water, or left for some time in water before examination. After being stained, the filaments display a dark-blue hue when examined under a microscope through a light blue filter, while other organs are violet-blue. With daylight illumination, much of this difference is lost, a dark blue color predominating. The modified Gram stain produces much the same shades.

The interpretation of observations is as important as is the staining technique. Here it is that Mangin seems to have been led into errors, and others have followed in his trail. They have confined their observations to an examination of the darker-stained margins of the side walls shown in Figs. B and C of Plate I, neglecting to examine the parts of the sloping sides of top or bottom walls, such as may be seen in about every other cell section. Fig. D of the same plate shows the same cell seen in Fig. C, but from above in the direction of the arrow. Cell angle "a" appears in both, and hyphae seen from the side in Fig. C are again seen, but from above in Fig. D. The dark blue hyphae which disappear at about the middle of Fig. C may now be seen in Fig. D to have turned back into the plane of the section and may be followed until cut off. Here is the explanation for Mangin's lamellae extending into the cell lumen; hyphae can bend in this manner, plates can not. Undoubtedly this artifact has been seen by other observers but has been deemed to be of no importance.

The hyphae in these diagrams deserve further study. The cellulose walls are unstained; the violet portion is the matrix (slime and pectin compounds) between the cells, plus the enclosed filaments (Mangin's lamellae). The relative thickness of the cellulose wall and of the layer of slime, etc., is indicated by their relative areas. The cellulose (or unstained) area is so narrow that the wall must be less than a micron in thickness, whereas the writer estimates the intercellular lining (which retains all stains to some extent) must be two or three microns in thickness in the parenchyma cells of the tubers studied. These fibers extend out

into the unstained zone, and must be assumed to be imbedded in it. Of course, a part of this unstained zone may consist of the mixed pectin-cellulose region of young walls described by Farr and Eckerson (1934).

The writer emphasizes Molisch's statement (1923) to the effect that no specific microchemical test has been discovered for pectin or pectin compounds, all tests for pectin being founded on staining and insolubility. Basic stains are retained by the lamellae. Ruthenium red is only a useful selective stain, not a microchemical test.

The position of the lamellae of Mangin and of the actinomyces fibers are shown diagrammatically in Figs. E and F of Plate I. The cellulose wall may be even thinner than is suggested in the diagrams since these filaments in surface view extend to the margin of the cellulose zone as seen in Fig. C.

Sections of potato or artichoke tubers stained with brilliant cresyl blue confirm the filamentous nature of these dark blue lines. The difficulty of photographing a twisting filament is evident to anyone who has attempted it, only portions being seen in any one plane (Figs. 13, 14, 18, and 23). On the other hand, all windings at all levels of a section may be reproduced in a drawing.

Figs. 2 (A and B) and 3 (A and B) are drawn from microtome sections of potato and artichoke tubers. In each figure, A is drawn at only one level (as it would show when photographed under a microscope), while B includes all filaments in the section visible at all levels. The general outlines of the cells may be seen in the former drawings, but the

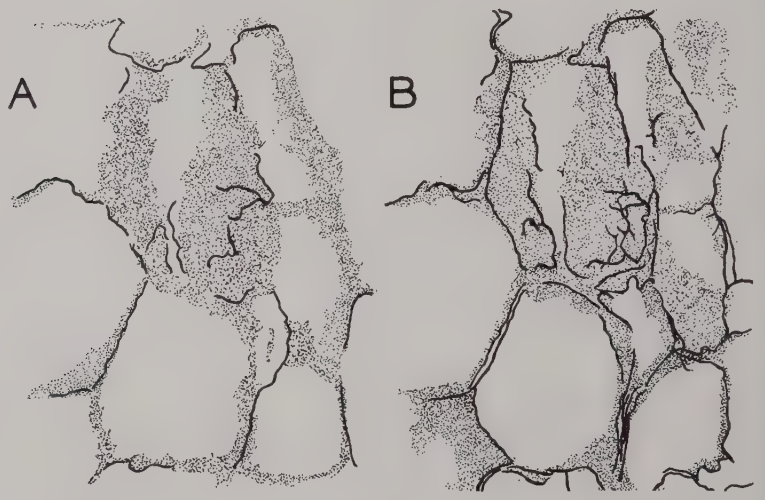


Fig. 2. A. Potato tuber section, 10 microns thick, drawn at one level, as in a photograph; cells chosen to show walls in the plane of the drawing. B. Same cells drawn to show all lines visible at all levels of the section. 250x.

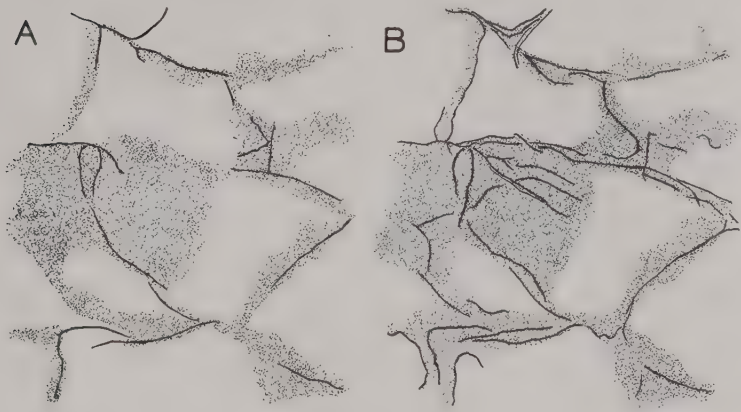


Fig. 3. A. Artichoke tuber section, 10 microns thick, drawn at one level as in a photograph; cell walls in plane of drawing also showing in part. B. Same cells with all lines visible at all levels of the section. 250x.

latter bring out many filaments that were not previously in focus. Cells selected for these drawings were so cut as to show a portion of the walls in the plane of the drawing. The filaments in the middle of the cells lie imbedded in these walls. They are more numerous at the cell margins because of the concave or convex bending of the walls at these places.

The calibrated fine adjustment on the microscope enables one to determine the vertical distance between two parallel filaments in the same wall. The writer would emphasize the fact that in focusing from one to another of the parallel threads, there is no gradual blending of the image at one focus into that at a different focus. One filament may be in sharp and brilliant focus, while a slight turn of the fine adjustment brings the second filament into clear view, but in a different position. This second line cannot be the wall of an underlying cell, since the vertical distance between them is only 7 to 8 microns.

The distance between filaments probably varies quite widely. The measurement of such distances when filaments are imbedded in a wall in the plane of the section would seem to be easier than measuring distances between filaments by vertical focusing. Few cell walls are even approximate planes. The walls invariably show more or less bowing; hence, plane end-walls or side-walls showing imbedded hyphae are rarely seen in sections, the bowing being sufficient to take them out of the plane in which the section is made. The composite twisting intercellular filamentous structures have nothing to do with the regular striations which seem to be a part of the composition of cellulose walls when they are photographed by x-rays. These latter cellulose markings appear as regular and spiral as a series of coiled springs in the photographs presented by Hock (1942).

Mangin worked with hand sections, in which planes and filaments might very easily be confused. No attempts seem to have been made to use microtome sections from paraffin-imbedded pieces of tissue. In microtome sections, two conditions must exist: (1) no wall can be cut without a middle lamella showing, many breaks sometimes occurring either part-way or all the way across a dividing line (Figs. 13, 14, and 18); (2) if these structures are lamellae, they should sometimes appear narrow, when the cell walls between which they extend are cut at right

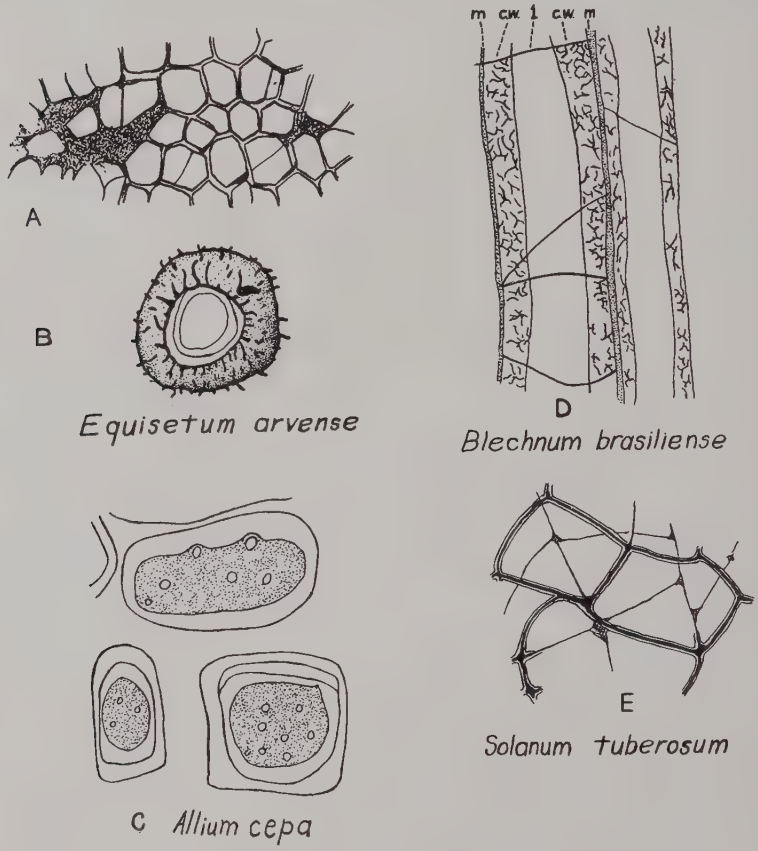


Fig. 4. Some of Mangin's figures redrawn. His technique is to draw part of the side walls as well as the cell contents and this is so unusual that it is a little difficult to interpret at first. The *Equisetum* figure B, that of *Blechnum*, and the *Allium* figures show (partially) the outside of the cell walls as well as the cell contents. In the *Blechnum* figure, *m* is the middle lamella; *c.w.* is the outside of the cell wall; and *l* is the cell lumen. In *Allium*, the outer line around each cell would be one of the middle lamellae.

angles, whereas at other times they should appear broad, when the cell walls are cut on a slant.

While Mangin's figures are correct, his interpretations are fundamentally wrong in many cases. Still, he must have had some basis for his observations and deductions. Perhaps his methods and line of thought may be retraced. He used only hand-cut sections in his studies of the wall structure and the finer details and their relationship had to be brought out by focusing the microscope. This may have led to mistakes in interpretation. The basic type-plants which he studied most carefully, and to which he compared others which he observed less carefully, were various species of *Equisetum*.

Equisetum contains large intercellular spaces into which parenchyma cells secrete little warts or excrescences onto an exposed wall. Sorauer (1909) shows such excrescenses on apple cells lining the seed cases, and many other examples could be cited. These wart-like growths and the layers inside them are generally thought to consist of calcium pectate. *Equisetum* at times shows this warty appearance (Fig. 4, B), the warts extending out as slender clubs (*bâtonnet*) which may be branched or which may be simply buttons (*boutons*). A thick wall connects them. Mangin's supposed outgrowths which he thought extended into the intercellular spaces of moss cells (*Blechnum*) (Fig. 4, D) may not have been the same structures. His drawings are correct but his interpretation may be erroneous. These growths were probably filaments of the type which the writer has described in this bulletin. They were imbedded in the intercellular materials and did not protrude into the intercellular spaces as Mangin suggested.

Inside the warty layer are other more or less concentric layers (Fig. 4, B), which are other lamellae. Mangin must have seen and drawn these from a series of optical sections by focusing his lens up and down. However, their presence would make the cell coat enormously thick. Parenchyma cells are always thin-walled but this did not seem to have troubled Mangin, since he apparently was unable to visualize just what adding these inner circles would do to the width of the wall. He described and drew these inner lines, which actually must have represented filaments lying in a series of planes, and therefore would not indicate a thick cell wall. Certainly they were neither plates nor laminae.

His drawings of the cell walls and of the so-called middle lamellae of onion bulb and potato tuber cells are much distorted, since they are a composite picture of a series of different focuses. Drawings of onion bulb cells show three or four lamellae (Fig. 4, C) with a small cell lumen. Such drawings could only have been made by including everything seen at a series of focuses. As reproduced, the onion parenchyma walls appear very thick, whereas they are known to be very thin.

His drawing of the walls of moss cells (*Blechnum brasiliense*) are similar in that they show part of the curved walls of the long cells with

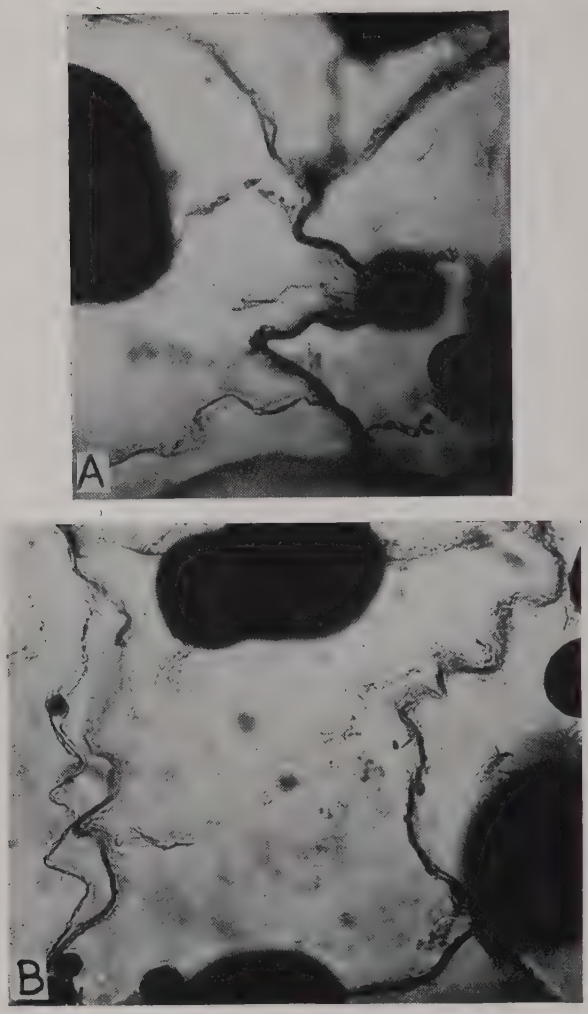


Fig. 5. A and B. Actinomyces filaments from mature potato tubers. These photographs show variations in the size of the filaments and their irregular, winding paths, partly in and partly out of focus on the side walls. Note the clear tubes, especially in B. 800x.

the irregular configurations which the writer would interpret as actinomyces strands.

His drawing (Fig. 4, D) and description of potato tuber cells also are derived from observations made at several focuses and are even more confusing. The description reads:

Deposits of calcium pectate filling the intercellular spaces. The accumulation of calcium pectate on the external surface of membranes instead of producing bodies with sculptured margins or laminate or filiform outgrowths may completely fill the intercellular spaces. Besides those of *Equisetum arvense,* the parenchyma of the potato tubers may be cited. A thin slice of this tissue freed from the starch grains which hinder observation shows between the cells a very narrow canal often filled with an amorphous substance which stains with the reactions of pectin. This substance, as narrow ribbons, fuses at the junction of a number of cells to form a star-like mass; these ribbons do not seem to fill the intercellular spaces completely but to have slightly shrunken from the sides.

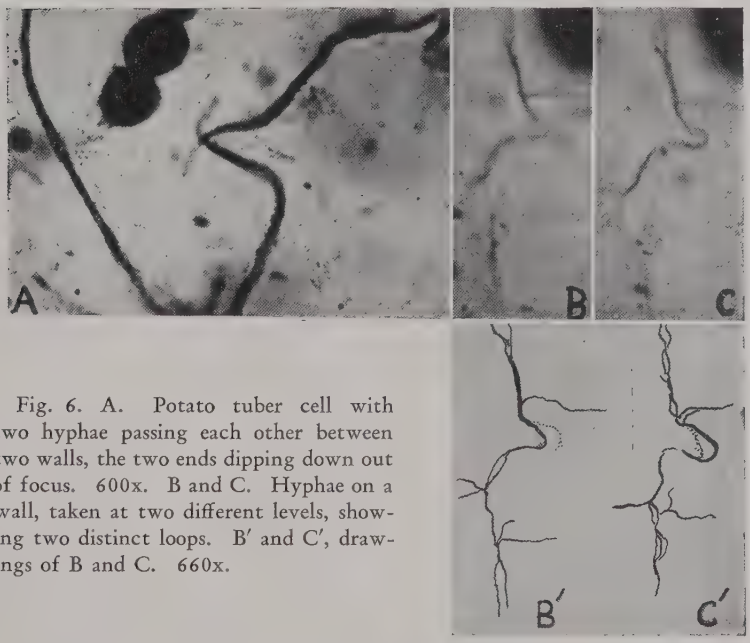


Fig. 6. A. Potato tuber cell with two hyphae passing each other between two walls, the two ends dipping down out of focus. 600x. B and C. Hyphae on a wall, taken at two different levels, showing two distinct loops. B′ and C′, drawings of B and C. 660x.

The first statement that the intercellular spaces may be filled with calcium pectate is not correct since the large and intricate communicating spaces contain gases with little pectin in any form. The intercellular matrix is not in the form of bands or ribbons though often, when stained with ruthenium red, it shows two dark red borders. The clear borders of Mangin's "ribbons" are not due to shrinkage, but are the cellulose or pectin-cellulose walls. Finally, the "ribbons" of the middle lamellae never unite at the cell angles, but branch, twist, and fuse as filaments, often at some distance from the cell corners.

Mangin's work should not be deemed the last word as to the structure and composition of the intercellular material. The *Equisetum* phases of his study, since they are the main basis of his conclusions and theory and are the ones to which he gave special attention, may be quite correct. The whole subject needs investigation with the use of modern paraffin-section technique, and it should be approached with an open mind. The

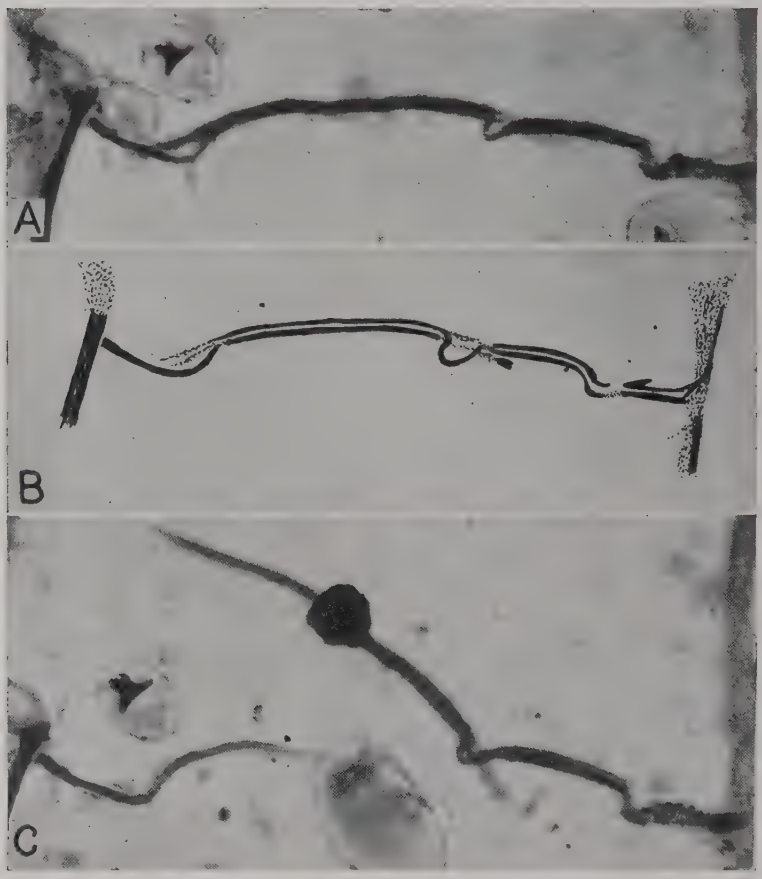


Fig. 7. A. "Cell wall" from tuber section stained with iron alum-hematoxylin. The dual nature of the dark line is clear in the branches, bends, and breaks of its components. B. Camera lucida drawing of the same "wall" to bring out the two components as they could be seen by focusing. C. In drawing a circle around this cell by the use of Winkler marking apparatus, the pressure of the diamond broke the elements apart into the form shown. The separation was only complete on the left; on the right, the two hyphae still adhered. A and C, 800x; B, 725x.

writer has tried to do this on the potato tuber, supplementing photomicrographs with drawings, since the camera and microscope lens function only in a very shallow plane.

The best pictures that could be taken are represented in Fig. 5, A and B, which show sections of old potato tubers stained by the modified Gram method. Of course, many of the filaments are partly out of focus, but the cells chosen were cut at planes represented by 3 and 6 in Fig. 1. Part of the sloping side walls are included, and it is between them that the branching and twisting hyphae appear. The broad, black lines are not single hyphae but are composites of two or more hyphae. Attention should be called to the clear zone around the small black hyphae. At the time these pictures were taken, this was assumed to be an optical effect, but later work has convinced the writer that the hyphae traverse the slime through a tube which they have cleared by digesting the material.

Fig. 6, A, is added because it shows two hyphae passing each other between two walls, although at slightly different levels. Such a passing would be a physical impossibility for two plates or lamellae, but could be readily accomplished by two fibers even in a very restricted intercellular space. Fig. 6, B, is another proof of the filamentous nature of these inclusions, which is very easy to demonstrate under the microscope and almost impossible to photograph in two dimensions. The two companion photographs (B and C), taken at slightly different levels, show two loops of two different filaments branching at slightly different levels. One of these loops does not gradually focus into the other; one is in focus, and then, with a short turn of the high power adjustment, the other loop (of a different shape) snaps into view. The drawings (Fig. 6, B′ and C′) may help to make this clear.

Fig. 7, A and C, were the result of an accident. Fig. A was photographed to show the filaments inside one broad dark "wall" of a potato tuber cell crossing each other inside the intercellular space. After this wall was photographed, the slide was marked with a Winkler marking apparatus. This machine draws a small circle by means of a diamond which is pressed down on the cover glass by a spring. The spring was pressed down too firmly in cutting the circle and when the slide was reexamined the components of the wall inclusions had separated. They were rephotographed to show the arrangement they had now assumed.

Fig. 8, A and B, show the arrangement and size of the filaments in the starch parenchyma and among the vascular bundles of a young tuber. Many of the walls in the starch parenchyma region are free from the filaments, but the filaments occurring between the cells of the young bundles are especially large. The Derrien-Turchini stain was used and sections, 10μ in thickness, were cut in order to follow the filaments along the cell walls. The intercellular material includes a small amount of protein, or at any rate it becomes a light gray with this stain. In this material is included some pectin, pectin-like compound, or slime which

takes on a light rose color with ruthenium red. In a photograph, the cell boundary is very dark since it is a summation of the darker-stained intercellular inclusions and a very considerable addition of lighter-stained

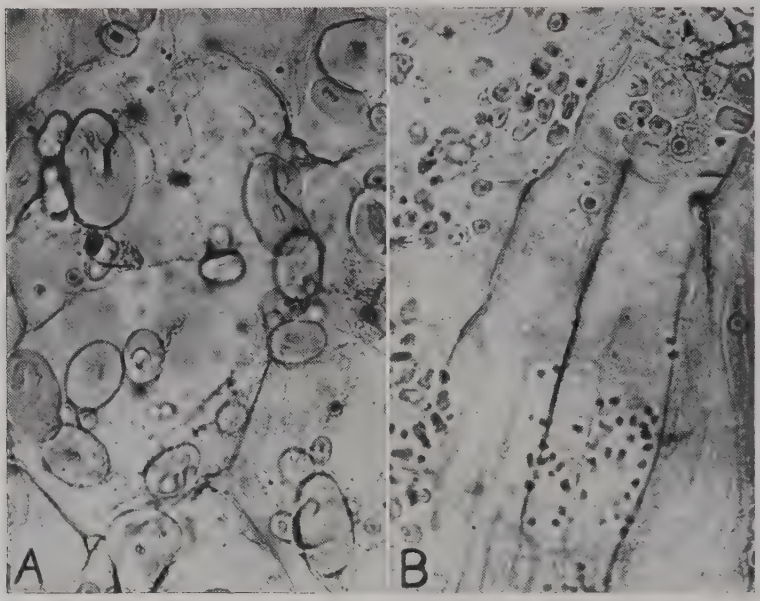


Fig. 8. Actinomyces filaments in young potato tuber. A. Between starch parenchyma cells. B. At side of bundle; note twisting path of the coarsest filament. 600x.

slime or pectin as the cell wall bends sharply down at the middle as in Fig. 9. In a photomicrograph, this combination (stained dark) appears

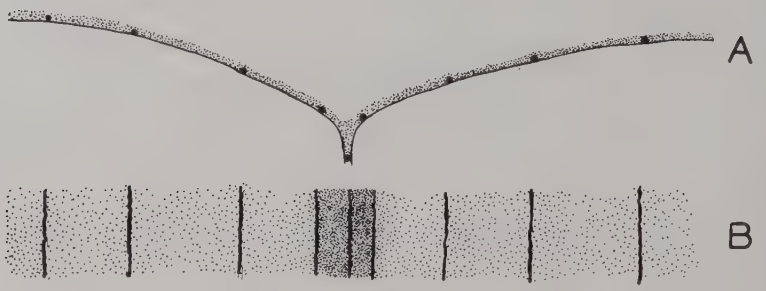


Fig. 9. Diagram of cell walls. Side view in A. View from above, B, shows the additive effect of stained intercellular materials and filaments in walls as they are seen from above.

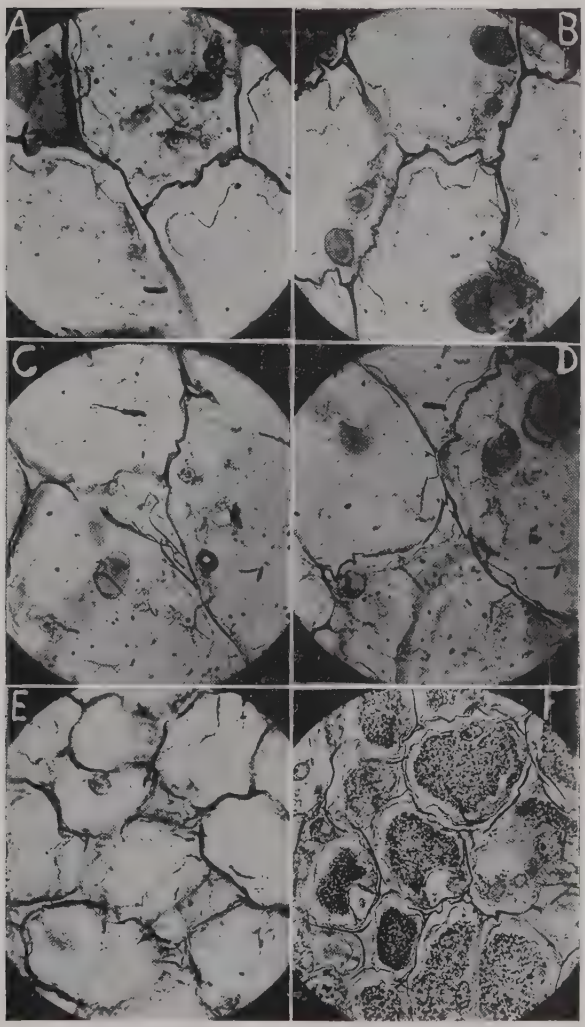


Fig. 10. Sections of potato tubers, stained by the Derrien-Turchini method (drawn as Figs. 11 and 12), to show the complexity of the cell peripheries. The broad, single lines consist of one or more finer and darker lines. C is probably the end of a cell. E shows a cell with much of the wall in the plane of the photograph but only a few of the filaments show in the photograph. F is taken from a cross section of an eye and the filaments are very large and numerous around these cells. 270x.

as one broad black line but on careful focusing, it resolves itself into at least two lines, as in Fig. 10, A, B, and D, drawn as Fig. 11, A′, B′, and D′. At some distance from these inclusions, frequently on the sloping side of the wall, is a part of another filament.

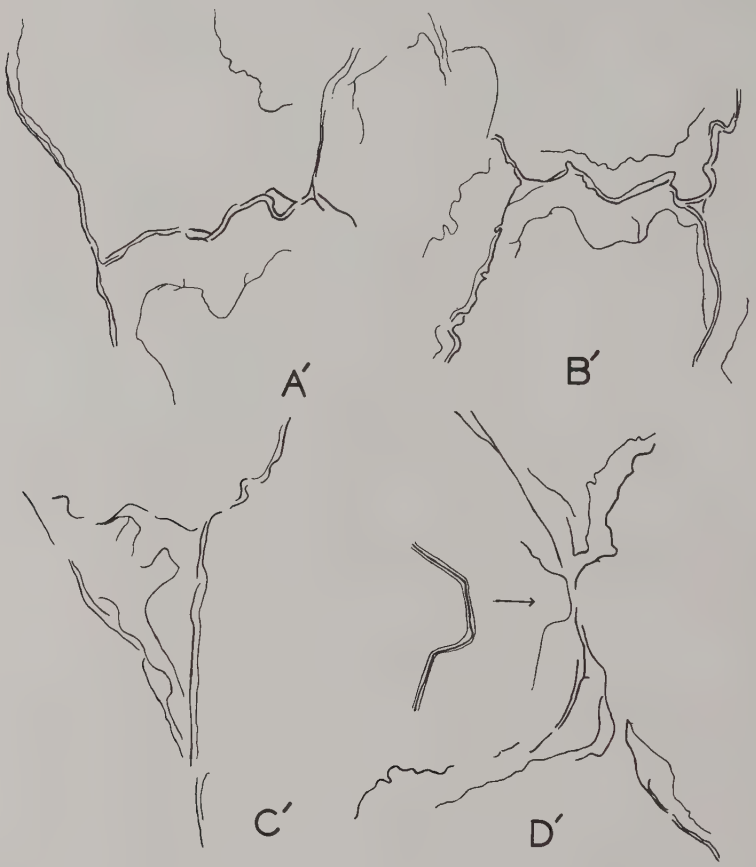


Fig. 11. Drawings of A-D of Fig. 10 to show the complexity of the potato tuber intercellular inclusions. Walls which seem to be a single broad dark line are composed of two or more filaments. In addition to these filaments, parts of another may be seen at some distance on the sloping wall in A′, B′, and D′. At the left in D′ is an enlargement of one of these side filaments in a clear tube. 500x.

The ideal place to view these inclusions would be on the cell wall lying in the plane of the section, but sections containing such walls are rare owing to the irregular form of the cells, the occurrence of even an end or corner in this plane being unusual. Fig. 10, C, however, appar-

ently shows an end or corner of a cell and Fig. 10, E, contains walls in the plane of the section with their inclusions although many of them are out of focus. They are, however, shown in Fig. 12, E′. These cells all have some walls that are nearly plane in part at least. Fig. 10, F, reproduces cells from a potato eye, near a bud. These cells are small and roughly spherical. The protoplasm is dense and granular. Its apparent distance from the cell boundaries is not caused by a shrinkage in fixation but is due to the fact that the walls of the cells, with their concentric inclusions slope steeply. The resemblance of these cells to those from

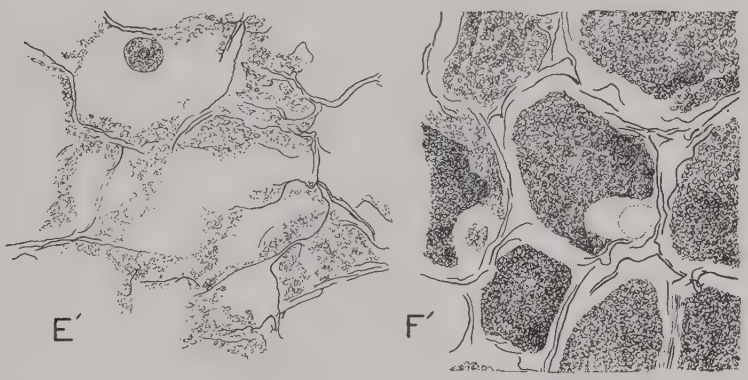


Fig. 12. Drawings of E and F of Fig. 10. E′ contains a cell cut to show much of the wall in the plane of the section. F′ reproduces cells from a potato eye, showing numerous concentric filaments between the cells. 400x.

the onion bulb drawn by Mangin (Fig. 4, C) is striking; approximately spherical cells being involved in both cases.

To sum up the evidence for the filamentous nature of these intercellular inclusions: they are like an actinomyces in size and in their very tortuous appearance. They are not like walls, but they do resemble hyphae between cell walls as seen from the surface of young potato tubers. They seem to bend into the cell lumens; appear and disappear on the slanting walls; and may bend and branch, and fray out into finer filaments. There is no morphological evidence to indicate that they are plates.

No chemical tests are available to determine definitely whether these inclusions are composed of pectin. However, filamentous shapes are not characteristic of chemical deposits in plant tissues, while layers or plates sometimes are. Calcium usually assumes the form of some type of crystal when it combines with a plant acid to form an insoluble salt. On the other hand, these twisting, fine strands are typical of the growth of mold and actinomyces hyphae. The results secured with the various staining techniques and the insolubility of the strands would indicate that they

are actinomyces filaments quite as much as that they are plates. The ordinary chemical technique is too crude to enable one to locate separately the two types of pectin, namely soluble and insoluble. However, Mangin's calcium pectate lamellae seem to be neither lamellae nor calcium pectate, but protein except in special cases.

Mangin's studies provided certain advances in our knowledge of the middle lamella: e.g., the usefulness of ruthenium stain, the lack of homogeneity in the intercellular matrix, and the presence of an irregular, warty layer in some cells, which is, probably, calcium pectate. His errors were due to his exclusive use of hand sections, his dependence on the microscope for determining cell wall structure, his neglect of the upper and lower cell walls, and his inability to reconstruct the third dimension. He should have examined his stained sections promptly, since the stain fades rapidly from the finer filaments. Fading is especially rapid in the cut horizontal cell walls. Within other walls the filaments are protected by two cellulose layers, and the stain fades less rapidly. Mangin's examinations were usually made on glycerin mounts, which may have been kept for some days, and he may thus have missed the unprotected and finely branched filaments of the top and bottom walls, and seen only the "lamellae" enclosed in the vertical walls.

The writer suggests that cytologists interested in the question of intercellular inclusions fix, imbed, section, and stain pieces of garden beet for examination. The beet serves the purpose better than the potato tuber since it contains no starch. The ruthenium red stain, 1-5,000, should be applied for about 10 minutes and then most of it drained off, the mount being examined at once while still in this very dilute stain solution. The cell angles should be carefully noted, for in them the filamentous nature of the darker red elements shows best. As a check, the stain recommended by the writer, brilliant cresyl blue in an aqueous 0.5 percent solution, should be applied to other sections for about 10 minutes, then washed off, and the sections examined in water. An oil immersion lens is not necessary since a 45x dry lens with a 10x ocular will show everything that can be seen without an oil smear. Not only should the cell angles be viewed but, also, cells in which parts of the horizontal walls are present. Such cells are shown in Figs. 31 and 35. The cytologist will at once say that these cells represent "poor fixation," but that is not true. These cells were selected to show parts of the horizontal walls. The filaments pass from the side walls onto these bowed slanting walls where the lens was focused on them. The third dimension can be seen if one knows how to look for it.

THE PRESENCE OF ACTINOMYCES FILAMENTS IN THE POTATO PLANT

In the Tubers

The discussion of the general problem has been largely based on material obtained from sections of young and mature potato tubers. The

filaments are large and especially numerous in the older tubers. The main reason for giving potato tubers first place in this discussion, however, is that the writer began his study of the filaments in such tubers in connection with his work on potato scab, and consequently had potato material available. At any rate, all underground plant organs are the easiest places in which to see the filaments.

In the Roots

The walls of all potato root cells, with the possible exception of those of the vessels, enclose many actinomyces filaments of various diameters. The cork layer (Fig. 13) is penetrated by stout actinomyces strands,

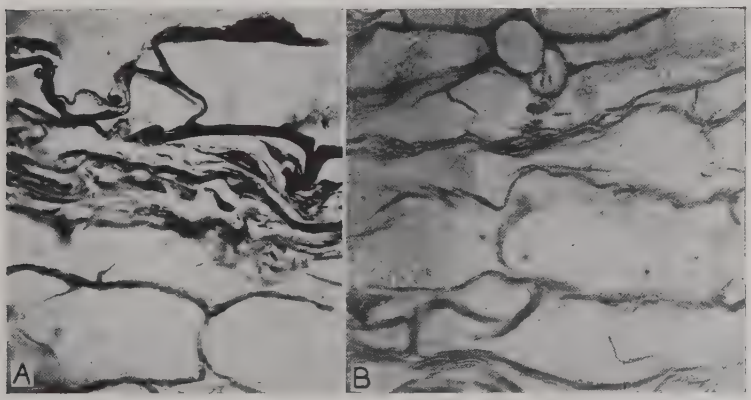


Fig. 13. Cross section of potato root. A. Cork and one cell layer of the pericycle. B. Cells of the pericycle; walls covered with strands, which make them a composite of fibers. 530x.

which are difficult to differentiate from the enclosing suberized and yellowish walls of the pericycle cells, of which there are two types. On the walls of the innermost cells of this tissue (Fig. 13, B), the filaments become more distinct and, two, three, or even more of them make up the thicker "walls." With ordinary staining methods, these filaments appear to be on a single plane, the strands composing the plane being blended together. When, however, a differentiating stain is used, the dye is removed sufficiently from the interfilamentous matrix so that the composite nature of the thick "wall" is revealed.

Filaments push between the cell walls of the phloem and cambium into those of the xylem. In the cambium, the hyphae twist irregularly and in places enter darkly stained, intercellular spaces where they cannot be traced. The hyphae are most abundant, however, in the pericycle. Fig. 13, B, shows double "walls" and the elaborate curves which the filaments make in cross sections of the root. Curious geometrical patterns appear which by no conceivable juggling could be considered cell walls.

Cell boundaries running from right to left appear as dark fragments but most boundaries at right angles to them show no darkly stained lines, and hence would be without "walls."

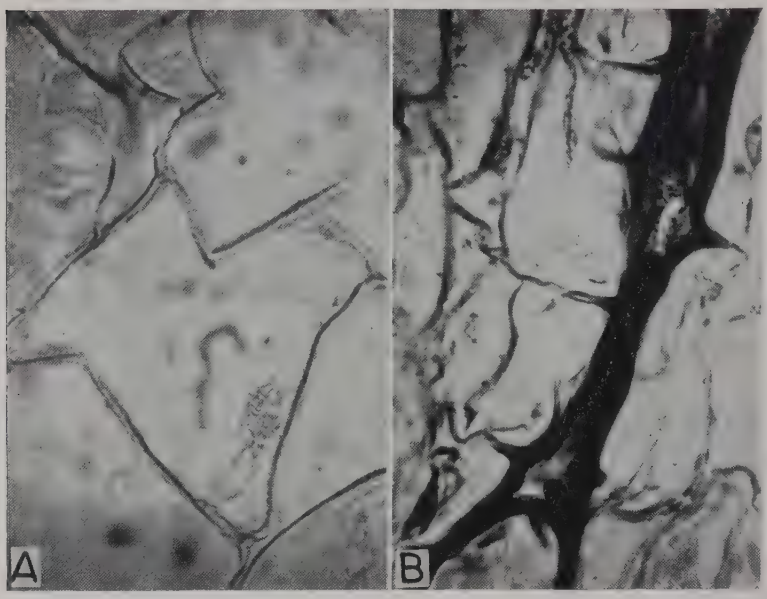


Fig. 14. A. Cross section of potato root showing distribution of hyphae on pericycle cell walls. Note two hyphae at right angles, but on one wall. B. Walls of the pericycle cells in longitudinal section enclosing filaments which run into a dark intercellular space. 680x.

Some of the unusual forms assumed by these filaments located on the wall may be seen in Fig. 14, A, taken from cells of the pericycle. The complex and unusual morphology of these strands is clearly indicated.

The theory that these intercellular inclusions are hyphae of an actinomyces is based largely on their relation to the potato tuber cells, as the writer has shown in a previous paper (Lutman, 1941). The large, split walls enclosing twisting filaments which stain the cytoplasm of the adjacent cells a dark brown were considered evidence that the infesting organism was a strain of one of the soil actinomycetes. Since these organisms are pectin dissolvers, no reason exists for their not finding an entrance into the roots of potato plants grown on scab-producing land.

Fig. 15 shows longitudinal tangential sections (A and B) and a cross section (C) of unfixed potato roots with superficial brown areas on their surfaces. Tangential sections A and B are at the margins of these discolored superficial areas. All these sections were cut by hand with a

razor from living material. In A, a brown area, which is discolored cytoplasm, is to the right of and below the letter A. In B, the brown area is to the right and the enlarged cell "wall" with a twisting hypha in it and the stained neighboring cells are clear. In the cross section, C, the browner areas and the twisting and numerous hyphae where the walls should be are evident. Fig. D is a small portion of C at a higher magnification. Some of the filaments were pulled out by the razor blade when the section was made.

The dark-staining filaments appear between parts of the walls and at the corners of parts of the cells near the tip of a young potato root,

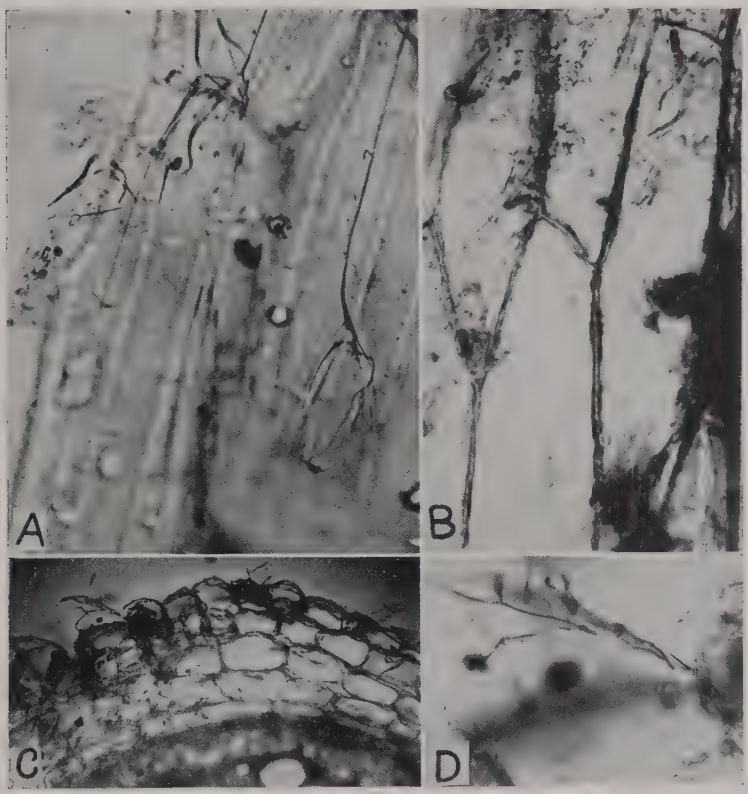


Fig. 15. Unstained sections of browned potato root to demonstrate appearance of filaments on cell walls, in part by their morphology and in part by the secretion of a brown-black pigment into the cytoplasm of adjoining cells. Sections A and B are tangential to the root surface, C is a cross section, and D is a magnified portion of C. The dark, branched mycelium in A, the thick, dark wall enclosing a filament in B, and the intricate walls of C are to be noted. A, B, and D, 700x; C, 150x.

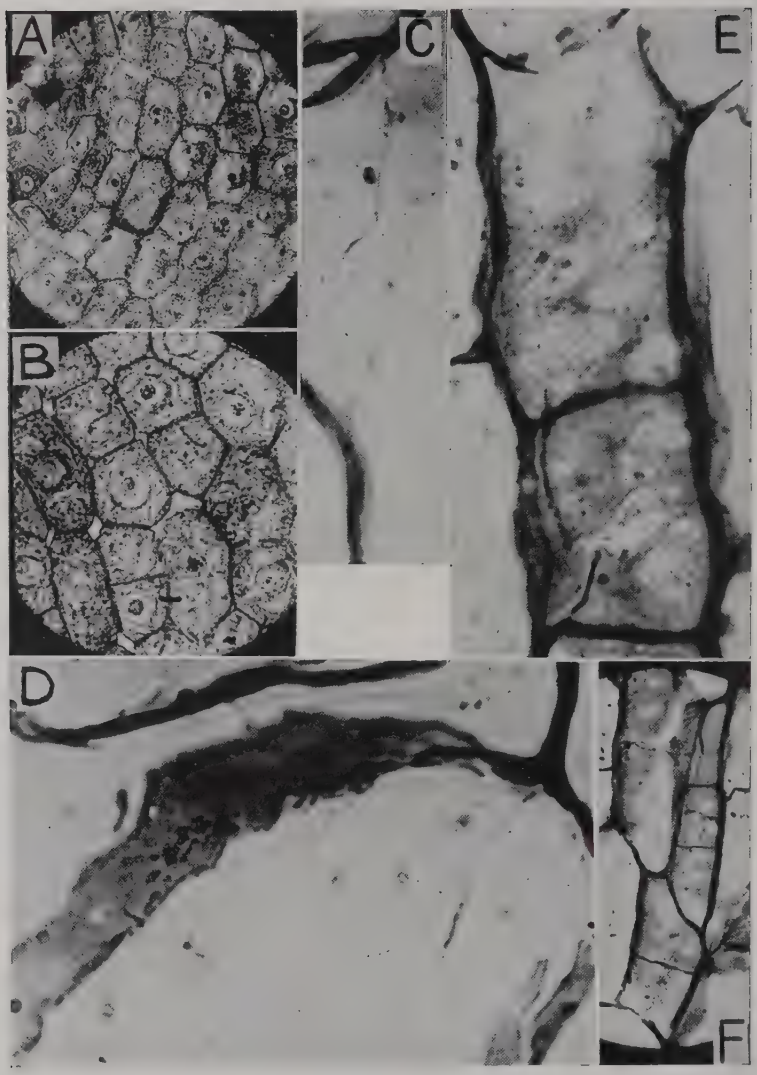


Fig. 16. A and B, photomicrographs of cross section of part of potato root cortex near tip (shown also as drawings in Fig. 17). Only part of the walls and intercellular spaces show the dark filaments. C and D, beet root cells with filaments emerging from darker-stained region, to cross part of the wall in the plane of the section (drawn in Fig. 30). E and F, carrot cells showing walls in the plane of the section with filaments and the clear tubes partly enclosing them (drawn in Figs. 30 and 33). A, 290x; B, 580x; C, D, and E, 1,000x; F, 290x.

(Figs. 16, A and B, and Fig. 17). These darker boundaries are not optical effects since they are occasionally double and sometimes bend into the body of the cell. At this stage, the cells do not seem to be surrounded by the filaments, as they are later. The filaments are most easily found and are most numerous in the cortex of the young root.

In the Stems

The tuber, being morphologically a stem, has essentially the same structure as the above-ground aerial, leafy stem, from which it differs in that it is located underground and is horizontal, shortened, colorless, much thickened, and without foliage. Of course, both types of stem may arise as sprouts from potato tuber eyes.

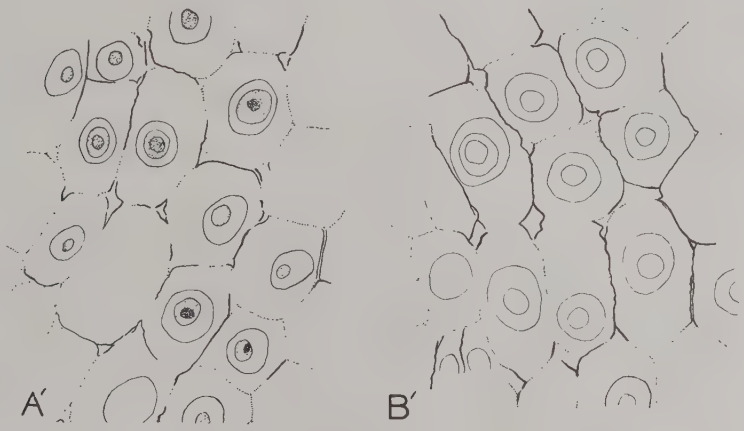


Fig. 17. Cross sections of cortex of young potato root near tip (drawings of photomicrographs A and B, Fig. 16). Cell walls bearing mycelium are shown as solid lines; others are dotted. A, 290x; B, 385x.

Sections of short, rapidly growing sprouts were found to be comparatively free from all actinomyces filaments. A careful search, however, disclosed irregularly branching bits of mycelium wedged into the angular, intercellular spaces of the small, rapidly growing cells of the sprouts. Apparently the sprouts had been able, by rapid growth and elongation, to free themselves from their slower-growing accompanying parasite (or symbiont), but never to become entirely clear of it. The tuber from which the sprout arose was very thoroughly infested, around every cell, and fragments of mycelium were probably carried out into the sprout. As growth slackened in the shoot, the slower-growing actinomyces was able to catch up and to fill the intercellular spaces and "middle lamellae." Infection from the seed piece would be so effective that no soil-derived infections would be necessary although such infec-

tions doubtless always occur each season. Soil infections are probably quite complicated, since many soil species and strains exist.

Young stems from half a centimeter to a centimeter in diameter were sectioned and stained with the gram-stain. Such sections are reproduced in Fig. 18. Photomicrograph A shows cortex cells. These cells look as if they might have been taken from a tuber without starch grains. The pointer at the upper right indicates a cell boundary which extends into a loop and then continues as a sharp bend at the cell angles. The dark lines which make up the apparent cell boundaries are not continuous, but are broken into bits which do not join.

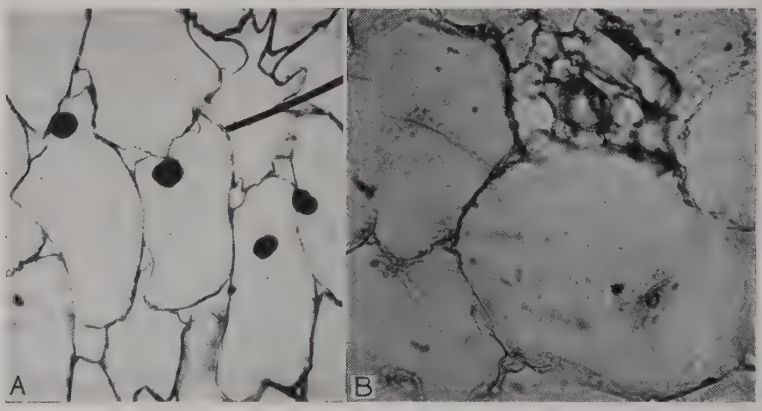
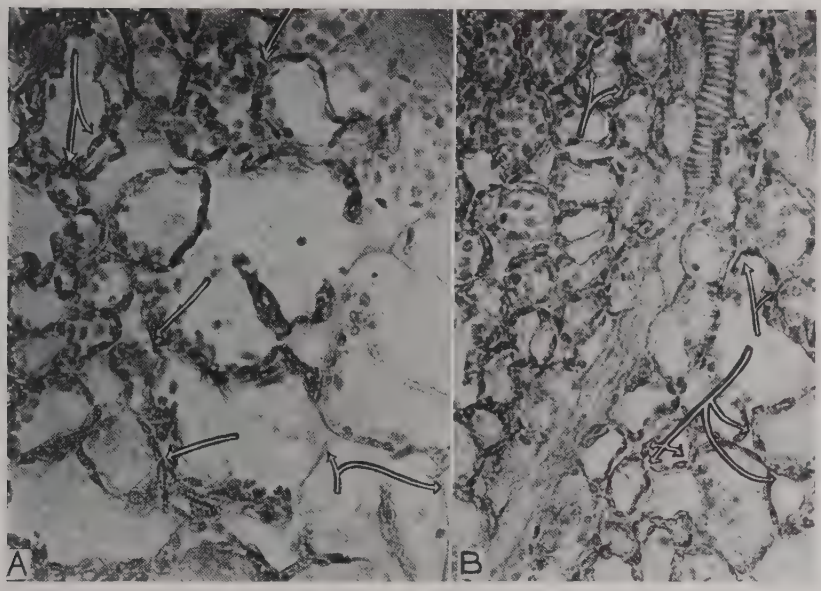


Fig. 18. Cross section of pith cells of young potato stem. A. Cortex with actinomyces filaments on walls. Note loops in cell angle indicated by pointer; also double nature of walls. B brings out crooked and intertwining strands on walls. End of a cell with many of the strands near the middle partly out of focus. 550x.

Fig. 18, B, represents a rapidly growing stem so stained as to bring out more of the details. This figure shows more clearly the complex nature of the cell boundaries, and the filaments which are partly in and partly out of focus. The confused tangle at the upper right probably represents a part of the side or end of the cell, the filaments being partly out of focus. The large cell just below the tangle of hyphae should show a cross wall but does not do so since no hypha was in focus.

In the Leaves

Actinomyces filaments may be most easily seen and followed in potato stems, those above ground showing them to better advantage than the tubers since the starch grains in the latter are at times a hindrance to following the cell boundaries. The leaves, being attached to stems, might also be expected to possess filaments.



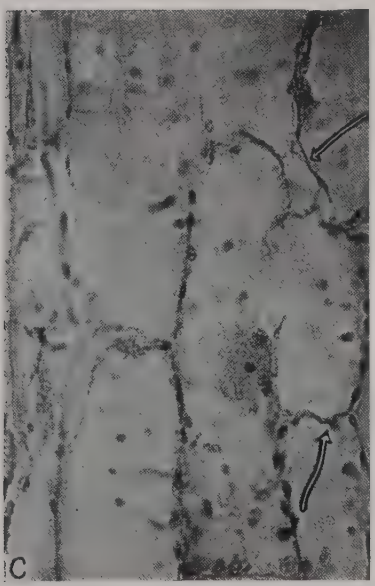


Fig. 19. Sections cut parallel to surface of potato leaf, modified Gram stain. Chloroplasts and cytoplasm are a red-violet and the fine strands of an actinomyces can only be differentiated by their dark-blue color. Fragments of strands, indicated by arrows, are most numerous near vascular bundles. 530x.

Young leaves contain a few filaments, located near the vascular bundles. Sections in the plane of the leaf show them to better advantage than cross sections of the leaf, since the filaments run parallel to the tube-

like components of the bundle. Cross sections would, therefore, only show them as short fragments. They are hard to find even when differentially stained, showing only as thin and short fragments between the cells. Furthermore, the intercellular spaces are large. Were it not for their dark-blue color, the filaments would be lost in the mass of reddish-purple chloroplasts and nuclei.

The mycelium is more abundant in older than in young leaves, and easier to follow in a surface view (Fig. 19). The filaments are very tenuous and are hard to photograph and to distinguish in the prints. The strands have been indicated by arrows in the three photomicrographs reproduced. Under the microscope the blue color helps to differentiate them. These filaments are found in the narrower intercellular spaces between the walls of cells which practically touch, but never in the large intercellular spaces.

Leafroll and the Leaf Actinomyces Filaments. The location of the actinomyces filaments almost exclusively along the vascular bundles of the leaf suggests their possible relationship to the disease known as leafroll. It seems to be established that no successful attempt has ever been made to transmit the leafroll organisms from a healthy to a diseased plant by direct human agency, but the disease organisms are readily transferred by sucking insects from the veins of the leaves.

What essential difference is there between the fine point of an injection syringe and the still finer sucking tube of an aphid? The steel or brass point of the syringe is stiff and inflexible and when inserted into the plant readily penetrates cell walls. In most experiments, therefore, the inoculum is probably pushed into a cell. The insect's proboscis, on the other hand, being slender and flexible, is inserted into the intercellular spaces and follows them to the leaf interior without penetrating the cell walls. Büsgen (1891) was apparently the first to observe and to interpret accurately this method by which sucking insects feed. Horsfall (1923) and Dykstra and Whitaker (1938) have shown that sucking insects which carry the virus of leafroll from infected to healthy plants extract their food from the leaf by the insertion of their long sucking tubes between the cells (where the actinomyces filaments may be found). In the sucking process, the tubes might become infected with submicroscopic fragments of leaf actinomyces. After lighting on a healthy plant, the insect could inoculate these fragments into the habitat of the parasite, the pectin of the "middle lamella" of the phloem leaf cells.

Two important properties of virus diseases must be noted:

1. The organism causing the pathological condition is particulate; i.e., one bit of the organism produces only one infection spot. Under favorable conditions, the inoculum grows slowly in a small area, but it may later spread.

2. The organism will not grow on ordinary media. If the real point of infection is between the cell walls, the presence of pectin compounds in the medium might make growth more successful.

An examination of the tobacco mosaic virus in a fairly pure condition by the use of the electron microscope with a magnification of from 24,000 to 30,000 diameters by Stanley and Anderson (1941) showed long, slightly curved bodies which varied in length and at times seemed to branch. They had the appearance of broken-up, fine actinomyces filaments such as might be seen on a slide when a culture of these organisms is broken up and stained. The percentage of the visible particles which are viable and infective is probably small.

The slender filaments of the intercellular actinomycetes are only a fraction of a micron in diameter, and they break easily into short fragments which could pass even the finest bacteriological filter. Nothing is known as to the size of the particles which are capable of reproduction.

Another factor that has been disregarded in studies of the spread of leafroll is the ability of aphids to find the phloem of the leaf vascular bundles. They are apparently guided by some chemical differences which they are able to sense. The phloem region is strongly alkaline, a fact known in Sachs' time, and Fife and Frampton (1936) have determined the phloem of the sugar beet leaf to be as high as pH 8.2 and attribute the feeding behavior of *Eutettrix tenellus* to this alkaline characteristic.

In the leaves, the actinomyces filaments are concentrated in the phloem region. Actinomycetes are, in general, very strong ammonia-producers, as noted by Krainsky (1914) and some strains may produce a pH as high as 9.2 on culture media according to Afanasiev (1937). The writer has corroborated these determinations on actinomycetes isolated from potato foliage humus by adding small percentages of colorimetric dyes to ordinary nutrient agar before inoculation. In the potato leaf, similar alkaline reactions could be expected in the vicinity of actinomyces strands and might well be the chemical guide by which the insect proboscis locates the best feeding region of the leaf, the phloem.

Net necrosis of potato tubers is simply the death and browning of the phloem of the vascular bundles (Artschwager, 1918, 1923; Gilbert, 1928). Large intercellular cavities are produced between the cells, and the actinomyces strands extend up to them, but cannot be followed further because of the dark-brown color of these cavities. From the activities of soil actinomycetes, their participation in the formation and coloring of the cavities might be suspected. Bawden (1932) has discovered from his staining reactions that "the deposits in the intercellular spaces are of a gummy nature, rich in pectin."

The observations of Sanford and Grimble (1944) that the cut surfaces of all tubers showing general phloem necrosis were definitely

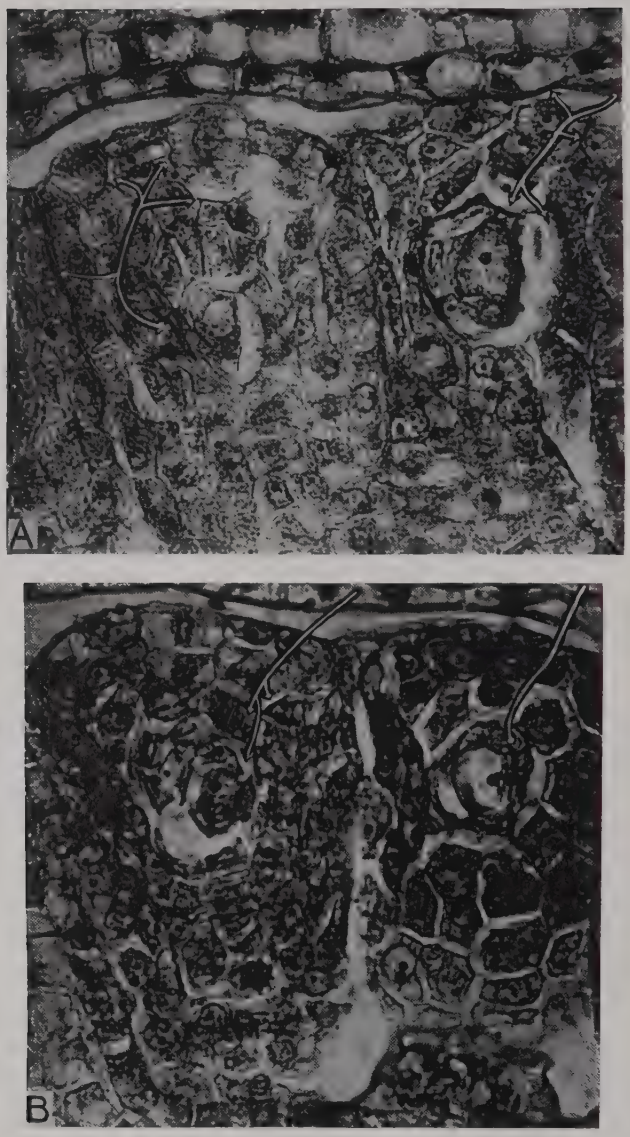


Fig. 20. Sections of potato ovaries showing egg mother cells surrounded by a plexus of actinomyces strands. Their dark-blue color is the best means of distinguishing them from the purplish-red cells. The actinomyces may be seen most readily in the ovaries in B. Strands of the actinomyces are indicated by arrows. 620x.

fluorescent fit into the actinomyces theory. The necrotic strands themselves were not fluorescent; the fluorescence was in the tissues adjacent to them. The investigators suggest that the tissues surrounding the necrotic phloem strands in the tuber contain a fluorescent chemical that apparently originates in the strands. The writer would be inclined to believe that the organisms are largely dead in the strands and that the fluorescent chemical arose from their destruction. The fluorescent properties of tuberculosis organisms are well known.

In the Flowers and Flower Stalks

The upward growth of the actinomyces filaments must keep pace with that of the organs which enclose them or they will be left behind and the tissues will in large measure free themselves from them. The flower stalks of most herbaceous plants shoot up with such speed during the growing season that it might well be that the slow-growing actinomyces filaments cannot keep up with them. If this were true, the flower buds might become relatively free from the mycelium which is so common in the lower stem.

Egg Mother Cell Formation. Sections of flower buds taken from a field of Green Mountain potatoes which had grown so rapidly that they had come into bud in June instead of July, as they usually do, showed the egg mother cells already formed (Fig. 20). The cell walls of the ovary as well as those of the ovules enclosed strands of an actinomyces. These formed such a network around the egg mother cell that the young plant and the seed could not have escaped infection, provided these flowers set seed. Seed formation, i.e., the formation of the small "balls," is now very uncommon in this variety. The chances are not one in a million that the flowers which were examined would have set seed, since no seed balls were found in the field. It may be assumed that the flowers dropped by means of an abscission layer in the pedicel (p. 46). If the flowers had formed seed, the seed might not have been infected by an actinomyces, and growth might have been rapid enough to enable them to escape temporarily the actinomyces filaments. If seed balls had been formed, they also might have been free from the actinomyces, as well as the enclosed seed. Seed balls, seed, and the young plants grown therefrom were examined with this hypothesis in mind.

Pollen Formation. Potato pollen grains are formed at exactly the same time as the egg mother cells. They never show a high percentage of germination, especially in the best commercial varieties. In sections showing fully mature grains at one end of the anthers, intact tetrads were seen at the other end (Fig. 21). Between the tetrads was located a faintly stained, light-purple substance through which could be traced dark-blue strands, apparently of an actinomyces (Fig. 21, A). Similar strands were traced between the tapetal walls. Around some tetrads these strands were twisted so tightly (Fig. 21, B), that they may have prevented the four cells from separating.

The Pedicel Abscission Layer. No detailed account of the formation and anatomy of the abscission layer in the pedicel is necessary. The external evidence of its formation is a slightly raised yellow ring about midway on the pedicel. Longitudinal sections of pedicels, fixed on the same date as the egg mother cells, showed external evidences of abscis-

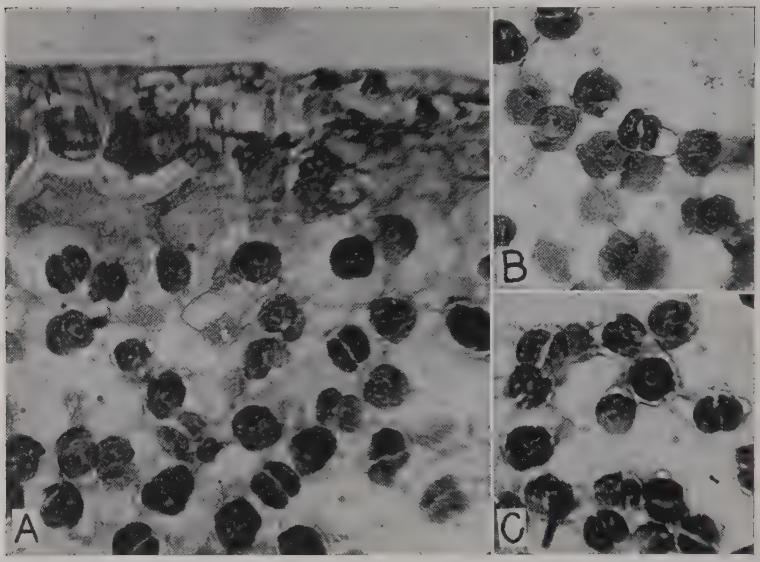


Fig. 21. Potato pollen tetrads. The tetrads are stained a dark reddish-purple and the slime between them a lighter violet, but surrounding and enclosing the tetrads are fine dark blue strands which apparently belong to an actinomyces. These strands cross the spaces between tetrads as in B; or may enclose a tetrad, shrunken away from them, in a mesh as in C. 800x.

sion. Kendall's account (1918) of the histology of the abscission layer in various *Solanaceae* was confirmed by these observations of the potato flower pedicels. The first evidences of the parting of the cells may be seen in the walls of those of the outer parts of the raised layer. Cell walls have been split apart and a dark material has filled the intercellular spaces (Fig. 22, A). Actinomyces filaments, stained a dark blue, traverse the walls of many cells of the pedicel and some can be traced into these intercellular spaces.

A cambium layer develops just a little later in the region of the vascular bundles (Fig. 22, B). Its intercellular spaces are smaller than those between the cells at the exterior of the pedicel but, just as with them, actinomyces filaments are frequently found on adjoining cell walls and may be followed into the intercellular spaces.

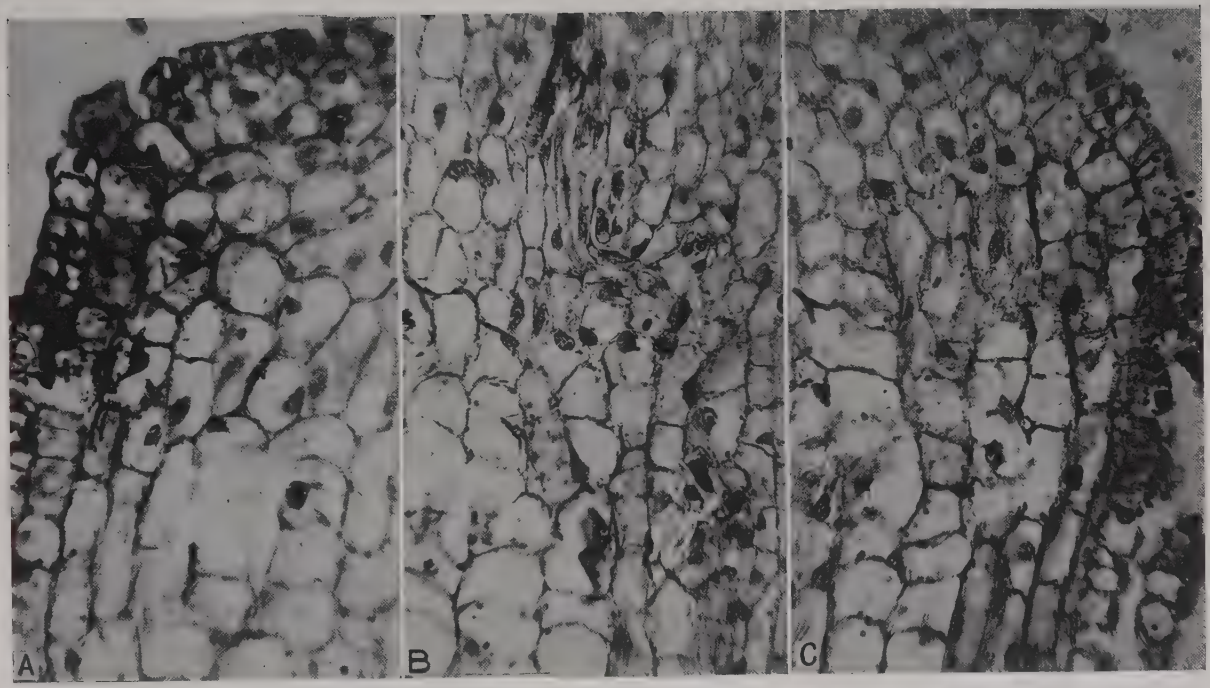


Fig. 22. Parts of longitudinal section of potato flower pedicel, showing beginnings of abscission layer, most advanced at sides A and C, less pronounced in the vascular bundle, B, where a cambium seems to be forming. Abscission beginnings are found in the enlarged, dark intercellular spaces filled with a stained secretion, especially between cells in A. Actinomyces filaments may be traced into these dark intercellular spaces. 330x.

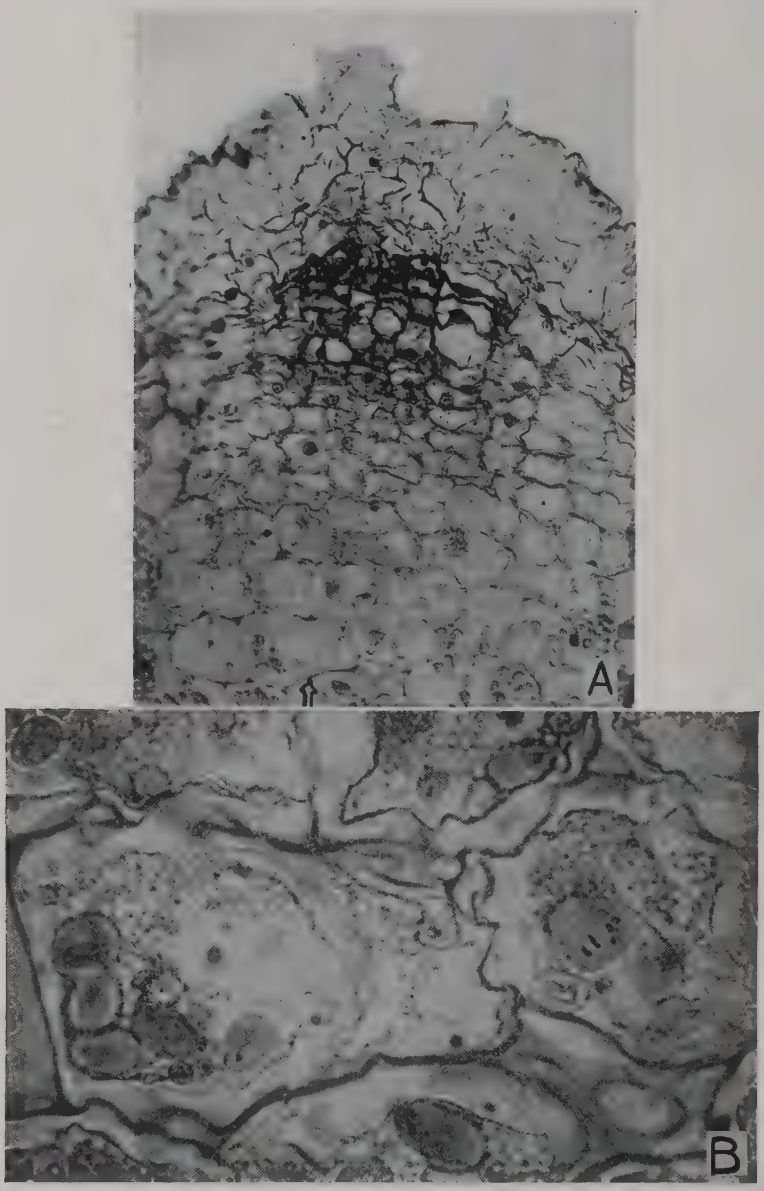


Fig. 23. A. Section of a lenticel of a very young potato tuber. The fungus shows only in the irregular, at times double, "walls" above and below the dark-walled cells. B. The cell above the arrow in A. This large cell is bounded by pieces of at least a dozen hyphae. A, 200x; B, 630x.

In view of the demonstrated ability of soil actinomycetes to dissolve pectin and to enlarge intercellular spaces, the writer suggests that these filaments, under the proper ecological conditions (such as excessive heat), become active, stimulating the formation of a cambium across the pedicel, enlarging the intercellular spaces by the dissolution of the cementing pectin of the walls, and so weakening the pedicel that it splits apart in this weakened zone, causing the buds or flowers to drop.

In Mature Seed Balls

Seed balls, mostly of the Katahdin variety, were obtained late in the autumn of 1941. They were still green although the seeds were mature. A few of the greener balls were fixed, imbedded, and stained. The cell walls of the pulp tissue which makes up their bulk were generally infected with actinomyces strands. The seed coats had developed and similar strands could be seen on the cell walls. Presumedly, young plants grown from such seed would become generally infected.

In Young Plants Grown from Seed

Potato seed from Katahdin crosses, carefully disinfected in a 0.5 percent chlorine solution, were planted in sterilized soil in sterilized pots, and kept under a bell jar until the plants had broken soil, when several were fixed, imbedded, sectioned, and stained. In every case, the thin-walled stem tissues were infected with actinomyces filaments similar to those found in older plants. These filaments must have been derived from the tissues surrounding the egg cell as noted above (p. 45).

Since all these plants were grown from seed obtained from crosses which had been grown in this country for many years, it was thought advisable to obtain seed from the original home of the potato. Through the courtesy of the agricultural station at Lima, Peru, seed gathered in the Andes valleys at Cuzco was disinfected and planted in sterilized soil. The resulting plants were infected with actinomyces filaments as completely as were the native hybrid plants grown from seed.

In Common Scab

The writer (1941), while studying the distribution of actinomycetes in scruffy and scabby potatoes, found that the filaments extended peripherally from the regions of abnormal tissue of the infected lenticels and the cork surrounding them, as well as centripetally into the tuber itself. These filaments, surrounding the cells as they do, must have pathological and biological significance. Infected and pathological lenticels (Fig. 23) show enlarged cells and thickened walls exterior to the cambium under the lenticels. All of those examined were infected with actinomyces strands, although many did not show the abnormalities seen in Fig. 23.

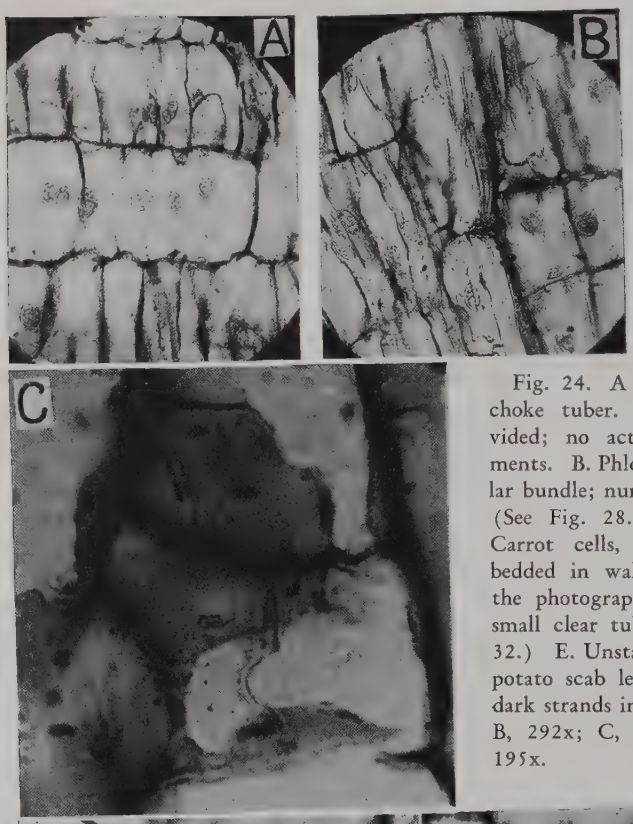


Fig. 24. A and B. Artichoke tuber. A. Cells divided; no actinomyces filaments. B. Phloem of a vascular bundle; numerous hyphae. (See Fig. 28.) C and D. Carrot cells, filaments imbedded in wall in plane of the photographs, enclosed in small clear tubes. (See Fig. 32.) E. Unstained section of potato scab lesion, numerous dark strands in the walls. A, B, 292x; C, D, 1,075x; E, 195x.

These superficial, tissue-infesting filaments in the cork discolor and thickened the cell walls between which they are enclosed. This clump of cells is made conspicuous by their thick brown walls, but all around them and under them are also located cells with infected walls which have not been thus browned and thickened. The actinomyces filaments cannot be seen in the brown-walled cells, but may be traced in the transparent-walled cells. The brown pigment in the walls of the conspicuous clumps retains the gentian violet color, thus masking the dark blue of the actinomyces. Their filaments do not stimulate the hypertrophy of any of these cells in the early stages but, later, irregular, large cells appear in the more mature scab spots.

The crooked lines which seem to bound the cells of the starch parenchyma must be carefully studied if their real nature is to be determined. In low power photomicrographs, many cell walls appear to be double, an intercellular space of some width separating the two lines. At times, instead of two crooked, winding hyphae, there may be three or more, which seem almost parallel to each other. Their composition can only be studied under a carefully focused, high power lens. A 45x objective with a compensating collar and a 15x compensating ocular giving a magnification of about 675 diameters without the use of immersion oil has proved satisfactory for such study. With such a combination, study of the cell walls shows that they are really composite structures made up of many irregular segments, which do not quite meet, and are interrupted by breaks. These breaks are not due to the fracturing of the "wall" by the knife in cutting, for, if they were, the two ends if brought together would just fill the break, whereas actually, if brought together, they would usually overlap. No photograph can bring out all this complexity; the only way in which the relation of the various crooked lines to each other can be really cleared up is by careful focusing and drawing. These filaments always seem to be largest and most numerous in the subscab tissues. Some notion of their number may be had from a hand section (Fig. 24, E) of a scab, cut thick enough to show the cells and their surrounding dark-stained inclusions. The numerous fine, branching actinomyces filaments are colored a dark brown and the same brown color has diffused out of them to stain the adjacent walls. No additional staining was necessary to make them visible.

In Cut Tubers Showing Cork Healing

Cork formation over the cut surfaces of potato tubers has been much studied because of its theoretical and practical importance. The writer (1926) discussed much of the literature on this subject from both a morphological and a physiological standpoint, and more recent papers are referred to in the general review of wound healing by Bloch (1941).

Sinnott and Bloch (1941), in a paper on cell division in wound

meristem, published excellent figures of this process. Their hematoxylin stain brought out filaments almost as well as the modified Gram stain, and since the filaments stained gray or black, they photographed to better advantage. Sinnott and Bloch interpret the lighter-stained walls as "phragmosomes" and as plates, but the dark lines which appear in some of them are clearly not plates but fibers, and are named "kinoplasmic fibrils." These fibrils invariably follow the phragmosomes, but their possible function and unusual morphology are not made clear.

The physiological factors involved in cork formation in tubers seem to be (1) moderate temperature, (2) moist atmosphere, and (3) three to seven days' time. The wound is first sealed by the dried remains of the outer cells, thus increasing the partially anaerobic condition in the deeper-placed cells.

If all potato tubers, whether scabby or clean, are always infested by an actinomyces, as the writer (1941) has found, a study of cork regeneration with special reference to the possible function of the actinomyces filaments seems advisable. The presence of numerous filaments of a foreign, invading organism must have some effect on the cells which they enclose. Their presence in the cork cambium may stimulate the formation of additional layers of cork until the tuber assumes a russetted appearance and the strands of an actinomyces under the lenticels cause the various types of corky scab, shallow and deep. While the writer's observations on scab offer no conclusive proof of the stimulating effect of actinomyces mycelium on potato tuber cells, they suggest that such stimulation may occur.

Methods. The ordinary methods of inducing this cork regeneration were followed. The drawings and photomicrographs (Figs. 25, 26) were made from material which had been cut for about a week and in which the regeneration process was nearly complete.

Observations. The histological details of this regeneration process having been carefully studied by other investigators, attention was directed principally to the distribution of the actinomyces filaments.

How a cut tuber surface is made impervious to gases and the effect which the resulting semi-anaerobic condition may have on the growth of the actinomyces filaments may first be considered. A single cell wall, much thickened by the dried contents of the outer cells, seems to be all that is needed to establish anaerobiosis (Figs. 25, 26). This outer protecting wall, which is much thicker than the normal walls, is impregnated and covered by some material which retains the dark-blue Gram's stain. Under this outer cell wall, the boundaries of the tuber storage tissue are filled with strong actinomyces filaments which become finer and thinner as they pass between the walls of the regenerated cork cambium. All the thicker filaments lie outside the layer of cambium cells, and from them finer threads extend into the new cork cambium.

The fibers of the actinomyces located outside the cork and phellogen seem to be as large as, or even larger than, those found within.

Actinomyces filaments are not only of finer texture between the cork cambium walls than are similar filaments located on the walls outside of it, but they are also even smaller than those found between the starch storage cells of the tuber. By careful tracing and focusing, they may be followed from the mass of the tuber through the phellogen and cork into the walls of the cells which lie outside the cambium.

Between the fibrovascular bundles of the tuber, the cork cells which have regenerated show a regular and easily followed distribution of actinomyces filaments on their walls. The tuber nuclei are large and seem to be rich in chromatin, indicating adequate nourishment and satisfactory growth, but the cork walls and the actinomyces filaments are very thin.

Fig. 25. A cork regeneration region of a potato tuber with a vessel stopped by a dark-stained plug; cambium fully developed under the dark outer layer. 350x.

If a vascular bundle is cut, the sealing-off process and the creation of an anaerobic condition are more complicated. The larger vessels may be plugged by a mass of dense material, which stains a dark blue (Fig. 25). The abundance of food in the bundle region leads to the growth of many new cells, which are not only more numerous but also much smaller than those in the regions not traversed by a bundle, and they no longer lie with their long axes parallel to the plane of the cut. The simultaneous growth of many tuber cells adjacent to the bundle, as well as the intrusion of many coarse actinomyces filaments into the same region, may in each case only be the consequence of a superabundant food supply.

The formation of new tissue from cells inside a tuber results in the simultaneous growth of an infesting actinomyces. Which is cause and which effect cannot be asserted on the basis of these observations, but the two processes are closely parallel. Food may be the determining factor for both, since tuber cell regeneration and numerous large actino-

myces filaments appear adjacent to the vascular bundles, where food is most abundant.

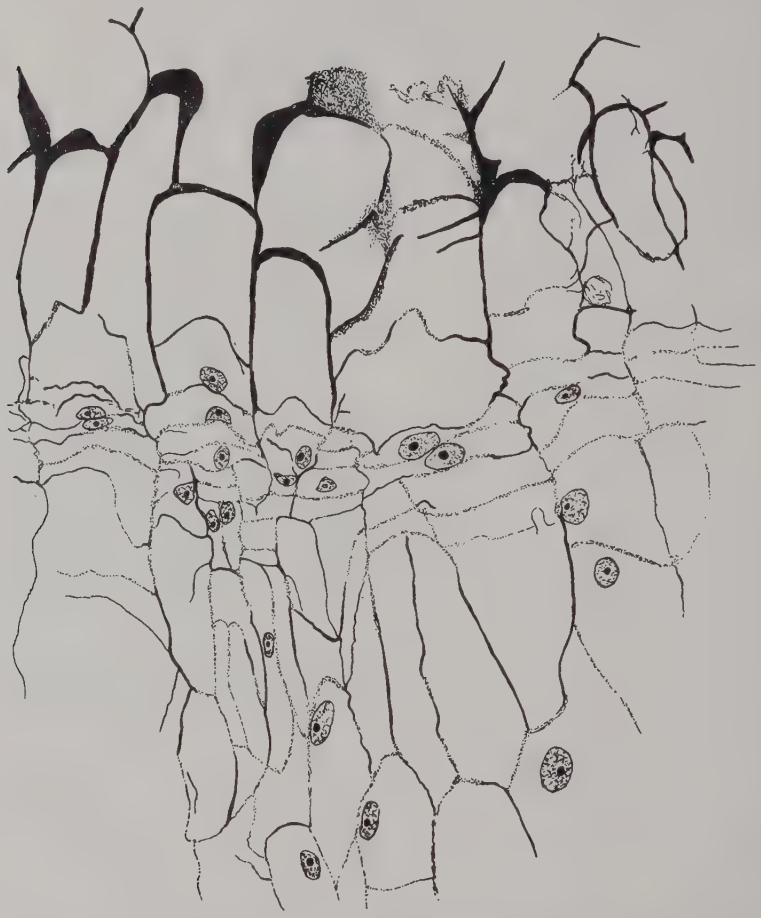


Fig. 26. Drawing of regeneration of cork over a vascular bundle of a potato tuber. The walls bearing filaments are represented as solid dark lines; the other walls are dotted. 400x.

THE PRESENCE OF ACTINOMYCES FILAMENTS IN OTHER PLANTS

In Jerusalem Artichoke Tubers

Artichoke tubers are morphologically similar to those of the potato, but are even more pronounced than potato tubers in their resemblance to branching, swollen, underground stems, with prominent buds and many eyes. Their internal morphology is also essentially the same. Cells are

delimited by similar "walls," which on close examination are found to be broken by frequent unstained parts where they fail to meet. These blue filaments are much larger in the outer cell layers of the tuber and become finer toward the center.

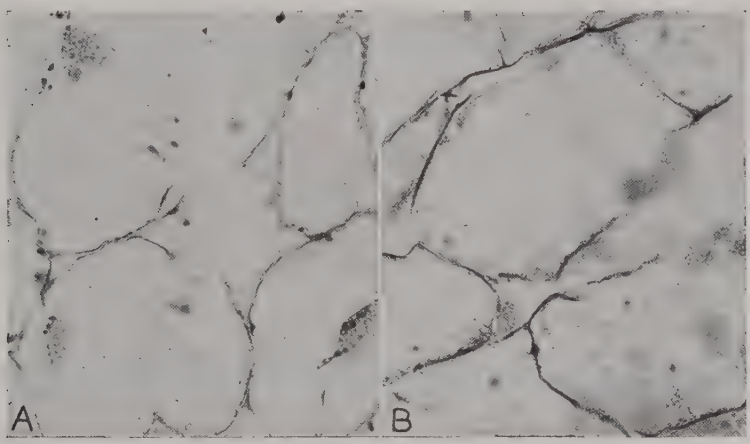


Fig. 27. Section of Jerusalem artichoke tuber. A. Thin-walled cells of cortex with actinomyces filaments shown as darker strands, double in some places, lacking in others. B. Cells of pith with darker, larger hyphae. 480x.

Mature tubers are covered by a cork layer, four or five cells thick, which is underlain by a cortex composed peripherally of large cells with thick walls and, in the interior, of smaller cells with thinner walls. Actinomyces mycelium in the cork layer is difficult to follow, the cells being filled with globules of a brownish-yellow material. The cortical cells under this layer also contain some of this material, but those of the interior of the tuber cortex seem to be free from it and here (Fig. 27) the mycelium is finer. The lines shown in the photographs are threads and not plates. The filaments are very delicate in the cortex (Fig. 27, A) and in the walls of the large pith cells (Fig. 27, B) which make up the bulk of the tubers. The slender blue filaments are so thin as to be almost unphotographable. The filaments are very abundant around the phloem region of the vascular bundles (Figs. 24, B, and 28, B′), running parallel to the long axis of the cells. In the cambium tissue scattered throughout the tuber, some of the walls are traversed by filaments but many of the younger cell walls are quite free from them. These conditions are comparable to those found in the cambium in wound tissue in potatoes (p. 51).

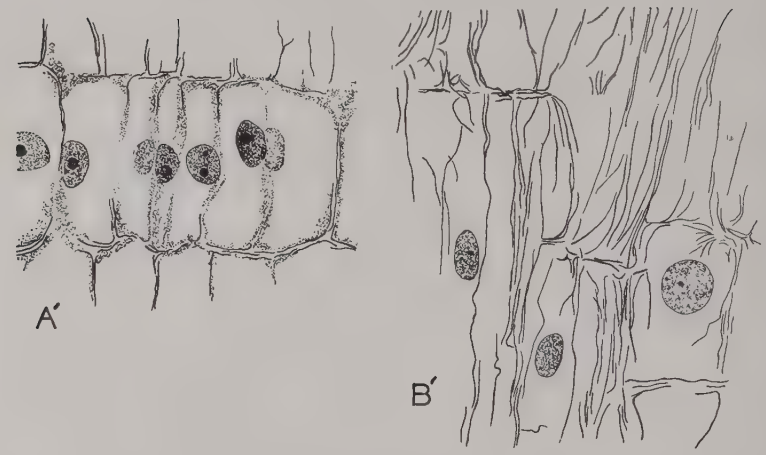


Fig. 28. Cross sections of artichoke tubers; drawings of photomicrographs A and B, Fig. 24. In A′, a row of cells have divided and the filaments have not yet intruded into the intercellular spaces of many of the cells. In B′, the filaments are very numerous in the long axis of the phloem elements of a young vascular bundle. 520x.

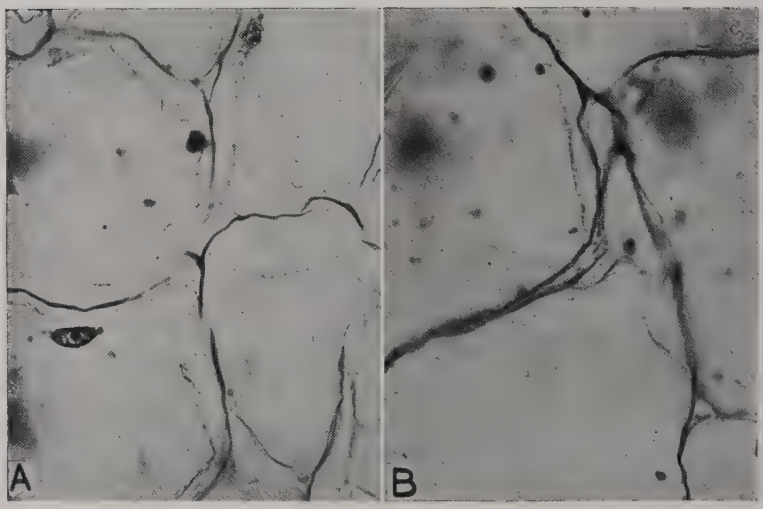


Fig. 29. Section of garden beet. A. Thin, regular mycelium is discontinuous and strands pass each other in places. The fainter lines near the darker strands are more delicate filaments on the bowed and slanting side walls and do not indicate plasmolysis. B. Angle formed by five cells with hyphae only partly shown, some being out of focus. 600x.

In Garden Beet Roots

In ordinary garden beet roots, sectioned and stained, the parenchyma cells which make up much of the root seem under low power to be quite

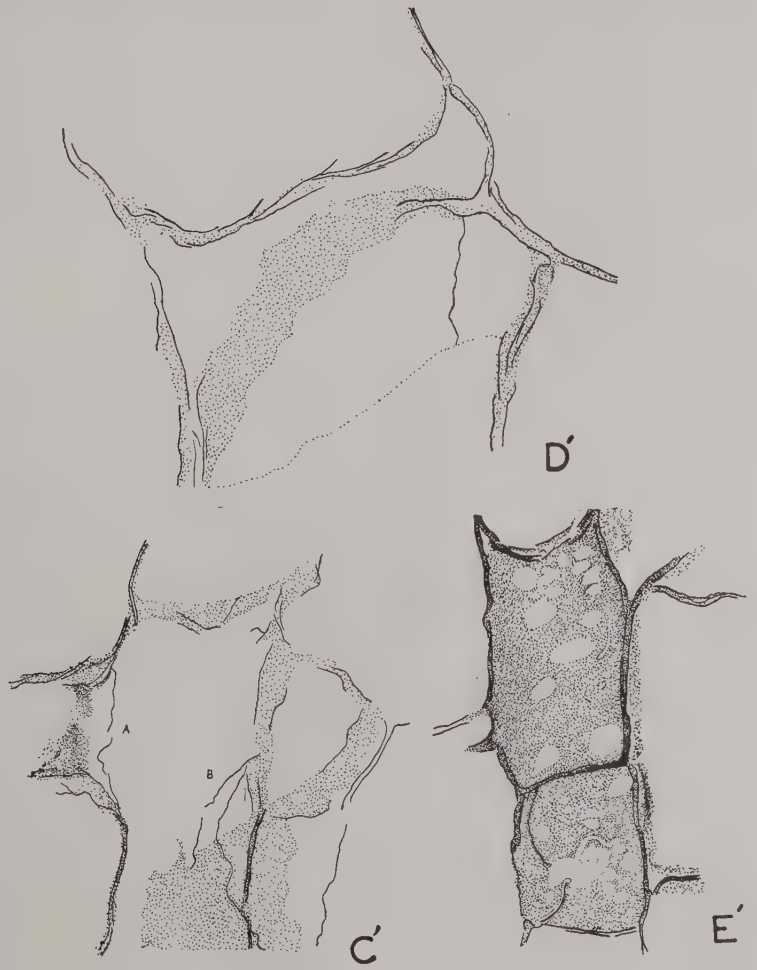


Fig. 30. C′ and D′. Cross sections of beet. Drawings of photomicrographs C and D of Fig. 16. In C′, the filament leaves the intercellular dark-stained region at A; a similar origin and emergence are shown on the other side of the cell at B; only part of the cell is shown in the photomicrograph. A similar attachment is shown in drawing D′ of Fig. 16, D. E′, carrot shown in Fig. 16, E, showing walls in the plane of the section and some of the imbedded filaments. The dark filament in the lower cell is partly cut off but the clear tube in which it was enclosed continues beyond the break. C′, 300x; D′ and E′, 450x.

regular in outline, with thin walls. A closer examination, under high power, shows the composite structure of some of these "walls" (Fig. 29), as well as their failure to meet at the corners. The twisting and intertwining of the filaments which compose them cannot be recorded by the camera with its one-plane limitations. Even with a sharply defining objective and careful focusing, the negative can only be suggestive and cannot take the place of an examination with the microscope and such focal adjustments as will permit one to follow the twistings of the actinomyces threads. No possible arrangement of cut planes could result in a figure where the "walls" of a cell go out of focus, separate, and pass each other.

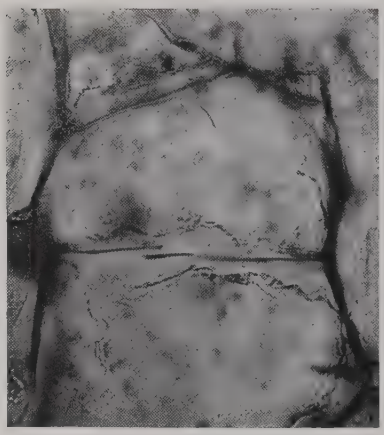


Fig. 31. Carrot root, cross section. Two hyphae passing between walls. Margins of the side walls are shown with numerous fine branches from the coarser mycelium spread on them. Note staining of cell walls by actionomyces. 690x.

Fig. 29, B, is probably as convincing a photograph as can be taken. Three or more strands of various diameters approach a cell angle to advance between the walls as intertwined hyphae. The delicate strands of hyphae may be traced from their intercellular origin out to the sloping sides of the cells.

The beet root, with its clear cells which are free from all starch, is excellent for studying these filaments, and parts of two other sections are reproduced as photomicrographs (Fig. 16, C and D) and as drawings (Fig. 30, C′ and D′). These cells were cut in such a way as to show part of the cell wall lying in the plane of the illustrations. The filaments originate in the intercellular region and wind over the sloping cell wall until both filaments and wall are cut off. The very tenuous nature of these filaments makes them difficult to stain and photograph, but the filaments sometimes appear to be located in a transparent tube, a situation also found in the carrot, as will be described later. As clear an example of this as has been found is reproduced in Fig. 16, D, and as a drawing, Fig. 30, D′. The thin dark filament originating in the dark intercellular space is continued over the cell wall, finally being cut off in the next section, and, of course, disappearing from this one.

In Carrot Roots

The swollen tap-root of the carrot is composed largely of regular parenchyma cells with thin walls. An occasional doubling of a "wall" in

places where it is very broad, the turning abruptly of a "wall" into the cell lumen, and a break in the continuity of the "wall" all indicate that here, as in the potato, filaments are everywhere present.

The filaments may be most easily seen at cell angles, where adjacent cell walls do not meet and fuse but pass each other at different levels. A good example of this was shown in beet cells (Fig. 29) but a different case appears in Fig. 31. The broader, dark-blue cell walls are always

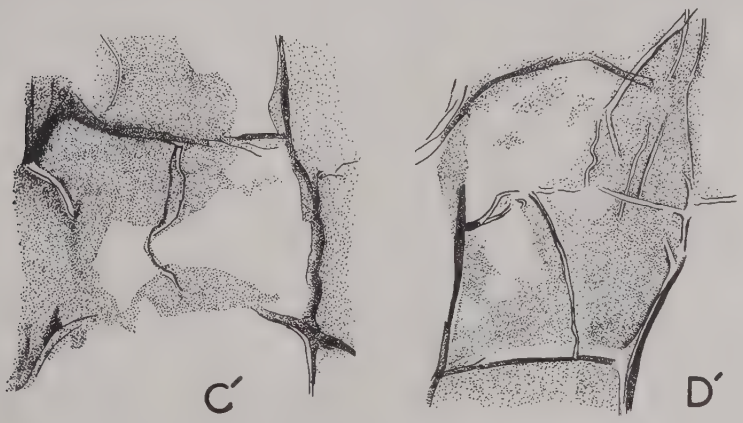


Fig. 32. Cross sections of carrot with much of the walls in the plane of the section showing (drawings of C and D, Fig. 24). In C′, the filaments are imbedded in clear tubes just outside the clear cellulose walls and partly in the granular intercellular material. Two tubes cross this cell; one only shows in the photomicrograph. D′ shows a tube and part of a filament extending entirely across the cell imbedded in the intercellular material. 450x.

found to be double with one "edge" at a slightly different height from the other, which runs parallel to it. In other words, a broad "wall" is composed of two filaments running parallel but at slightly different levels on the same carrot cell wall. The special reason for reproducing this figure is to call attention to a "wall" extending from right to left at the center. Breaks in a cell wall might be ascribed to action of the microtome knife, but this explanation would not fit here since the two ends of the broken "wall" pass each other. The very fine, twisting strands which seem to extend into each of the cells are finer branches of coarser mycelium imbedded in slanting walls. This represents neither poor fixation nor shrinkage. The top or bottom walls in the plane of the photograph bend enough so that a thin slanting slice of them appears with their included filaments, but only around the cell peripheries.

Sections of cells showing the wall in the plane of the section are seldom seen. Fig. 24, C and D, represented by Fig. 32, C′ and D′, shows several

cells in which part of this wall can be seen. The middle cell of Fig. 24, C, shows the filament emerging from the dark intercellular region, extending downward, and then being cut off by the upward bulge in the cell wall. This bulge is not enough, however, to cut off the clear tube in which the filament is enclosed. This clear tube is not the end of another cell wall, for another similar tube joins it, seen faintly in the photograph because at a different focus level but shown plainly in the drawing. Fig. 24, D, shows cell walls in the plane of the section, but many of the filaments are out of focus; in the drawing (Fig. 32, D′), however, they are all shown. In Fig. 24, D, a filament and its clear tube are seen crossing the middle cell. Toward the upper portion of the cell, the dark-stained filament can be distinguished. Below it and extending directly from it is the clear tube from which the lower portion of the filament has been cut off.

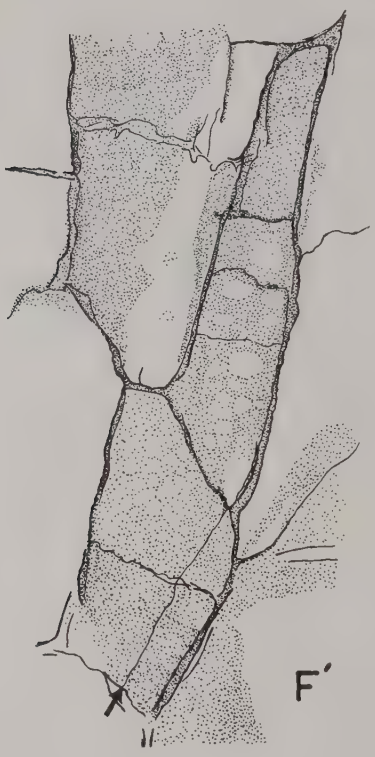


Fig. 33. Cross section of carrot, drawing of photomicrograph F, Fig. 16. A very delicate filament is seen crossing the lower cells. The walls in the plane of the section are irregular in contour, as are those in Fig. 30, E′.

The continuance of a "wall" from the outer cell boundary to the wall in the plane of the photograph can be seen faintly in the two lower cells of Fig. 16, F, and more clearly in the drawing, Fig. 33, F′.

In Parsnip Roots

The garden parsnip is provided with a cork layer three or four cells in thickness, and many of the cell walls enclose many actinomyces filaments (Fig. 34). The "walls" of the cell at the left of Fig. 34, A are all distinctly double, the strands of the mycelium lying parallel and so nearly at the same level that they could be photographed in the same plane. This is an unusual occurrence, for one filament usually lies a little below or above the other. The focus may be shifted from one filament to the other by changing the fine adjustment, usually from 8 to 10 microns, as marked on the drum on the side, indicating that the filaments are about this distance apart in the cell walls. At about the mid-

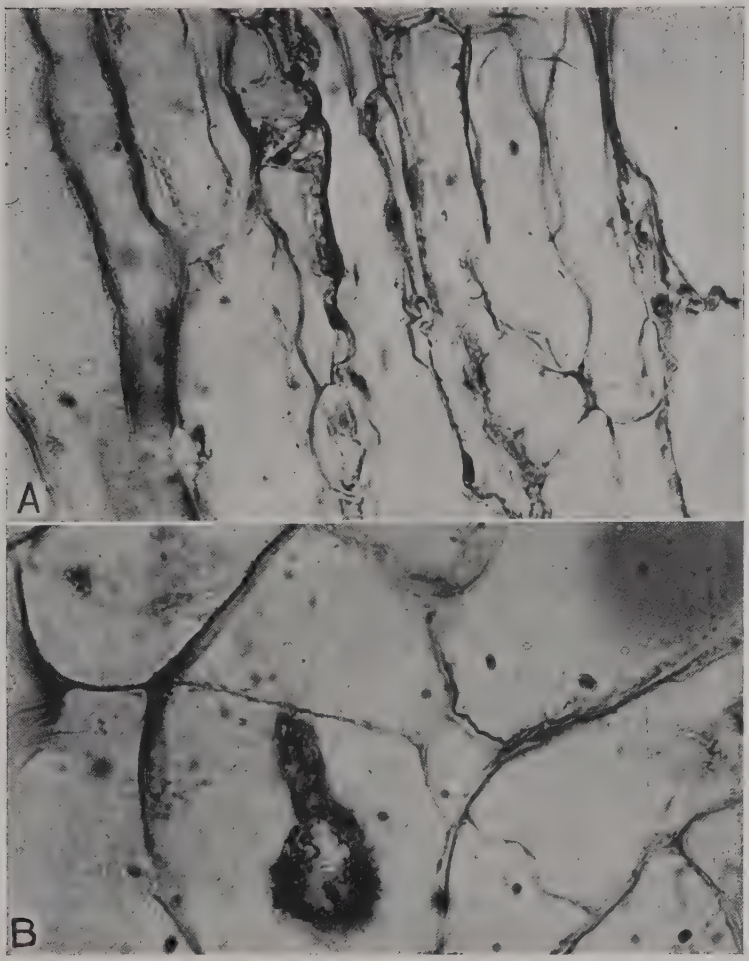


Fig. 34. Cross sections of cork layer of parsnip root. A. All cell walls infested by an actinomyces, parallel hyphae being seen in some cases. B. Cell walls with dark spaces between them at left; coarse hyphae and twisting hyphae on wall at center. 520x.

dle of Fig. 34, B, can be seen a twisting, irregular filament at the cell angle which disappears into other filaments which run parallel to each other.

Broad, dark regions between cells, seen particularly in Fig. 34, B, represent intercellular spaces containing one or more filaments imbedded in a darkly stained matrix, the dark irregular triangles at the left of this figure being the angles at which several cells come together.

In Turnip Roots

The enlarged tap root of the turnip is largely composed of regular parenchyma cells with such straight walls that actinomyces infection would not seem likely. Only a critical examination of the dark-blue lines which seem to bound the cells will show that they are a complex of filaments and are not plates. On the turnip cell walls, these lines are especially thin (Fig. 35) and thorough search is necessary before places can be found where the filaments separate into two or more strands.

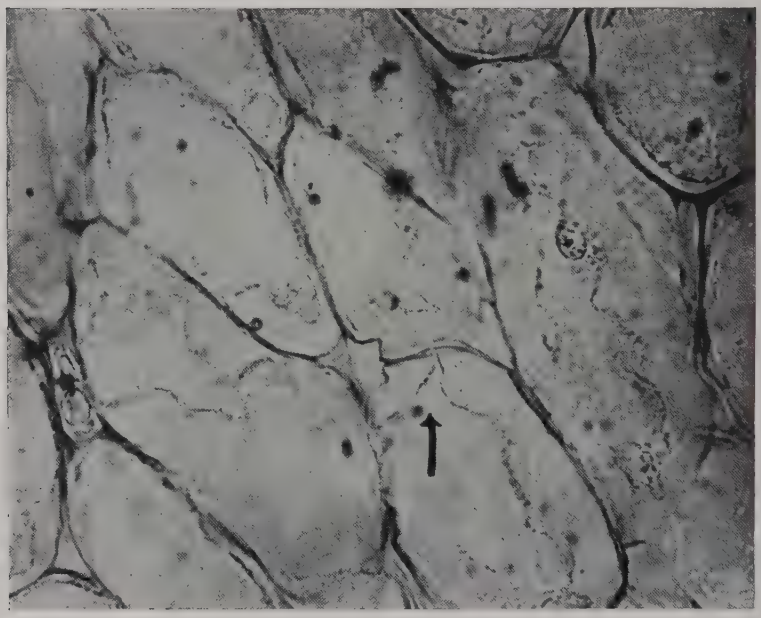


Fig. 35. Cross section of cortex of turnip. The cells are oval in section and much of the bowed side walls can be seen. (Compare with Nos. 1 and 6 of Fig. 1.) The filaments twist and cross between the walls but separate at the cell angles. They may be followed at the cell angle and on the side wall (see filament marked by an arrow). 520x.

For some unknown reason, the actinomyces strands in the turnip are comparatively straight and do not twist and turn as in the underground parts of the other plants studied. A region must be selected inside turnip cell walls where two parallel filaments lie so nearly in the same plane that they will appear in one focus, as shown in Fig. 35. The places best suited for examination are the broader, darker "walls," conspicuous by their apparent thickness and the dark stain. These "walls" will be found to be bounded by two parallel hyphae which separate and may branch

at an angle where three or more cells come together. The intercellular, angular space formed here may be quite large, as seen in the upper part of the photograph. The twisting of the hyphae on the walls may be seen. The sharp angle made by a hypha in a cell angle at about the middle of the photograph, and the twisting hypha in the wall of the cell just above it, should be noted.

These cells have not been plasmolyzed. They are the best examples shown in this paper of the sloping walls discussed on page 8. Not a single plane wall can be found, but every cell shows parts of the sloping side walls. These sloping side walls are usually clear (giving rise to the misinterpretation that plasmolysis has occurred), but over them travel the filaments which have their origin in the intercellular space. In Fig. 35 an arrow points to such a filament, which is partly in the intercellular zone and partly emerging on the side wall. All these twisted strands, which are partly in and partly out of focus, are filaments of an actinomyces. They are most readily seen at the cell peripheries but also appear twisting out over the sloping side walls.

Discussion

As previously stated, slides of stained sections of potato tubers were sent to a number of plant pathologists and histologists. Many of them examined the preparations and made comments to the writer. Since they presented many different viewpoints, they were helpful in a review of the writer's previous statements that actinomyces filaments were common to all potato tubers, clean or scabby, and that they were between all tuber cells. Much of this bulletin represents an attempt to answer the objections of these botanists. The only comment that has not been answered is that of a botanist who held that the broken walls and wavy edges of the cytoplasms were artifacts resulting from poor fixation, that the cell contents had shrunk and the edges of the cytoplasm had folded or rolled up in such a way that the margins resulting from such shrivelling simulated the windings of an actinomyces filament. As has been pointed out, however, the branching filamentous structure of these inclusions and the difference in pH which they show as compared to the enclosing substance would seem to make it fairly conclusive that they were never a part of the normal cell but are some sort of an intruding organism.

No one has ventured the suggestion that a new, and hitherto unknown, organ of the cell was found between the walls. Such a possibility should not be excluded, but the presence of such an organ is not very probable.

The most important feature of the invasion by actinomycetes of the intercellular region of most, if not all higher plants, including the ferns and mosses, is the general infection of all parts of the plants and its con-

tinuance from one generation to the next through the young embryo. The bulk of the mycelium of an actinomyces is to be found in the underground roots and tubers, but strands extend into the stem and even into the leaves and flowers. The strands are least numerous in the rapidly growing tissues. Whatever the biological activities of these invading organisms may be, they are most important in the roots and tubers and least so in the above-ground organs which have escaped infection.

The presence of a few scattered strands of actinomycetes in the growing points of buds and roots or in the piths would make little or no difference in the life of the plant. The slow-growing actinomycetes are outdistanced in the parts of the plant which shoot up very rapidly during the best part of the growing season, such as the flower stalks of the herbaceous plants, but as a tissue matures, its intercellular regions fill with actinomyces hyphae and the exact location of the enclosing walls cannot be accurately determined.

The sharply defined, geometrical figures of cells with their walls meeting in definite angles, such as those described by Marvin (1939, 1944) in the pith cells of *Eupatorium,* are lacking in all the tissues of the potato plant, even in the stem pith. The most nearly geometrically perfect cells are those in the rapidly growing shoots and sprouts, on the walls of which actinomyces filaments are uncommon or are of very small size, the growth of the organs being sufficiently rapid to leave the organism behind. Hence, the walls are regular and the angles are still clearly defined at the cell corners and are not rounded off as they become in old cells.

Wiesner's theory (1886) that cell walls are living and growing so long as they are surrounded by protoplasm, a dermatoplasm taking part in the growth and functioning of the walls, has been relegated in recent reviews (Heyn, 1940) to a minor position. Wiesner's microchemical test for cell-wall proteins, as well as the tests of his student, Krasser (1886), have not been included in recent microchemical compendiums of methods. But if the writer's observations and interpretations are correct, Wiesner's theory and tests are in part justified and verified, but with the modifications that the living parts of the walls or of the intercellular region are not a dermatoplasm but actinomyces filaments.

The early theory of Sachs and Unger that the walls and not the cell lumens convey liquids is literally true, if the theory presented in this bulletin is valid, for nutrients would have only to find their way into the actinomyces filaments through the cell walls of the root or stem to have a clear path with no interrupting walls to check their transfer. The vessels might serve as reservoirs, but the intercellular spaces with their actinomyces filaments would be the important organs for the interchange of materials. The living wall theory of the rise of sap is still upheld by the experimental and theoretical work of Ursprung, one of the leading plant physiologists (Beck, 1942).

A number of problems are suggested but are unanswered by this bulletin. One of the most important of them relates to the number of species of actinomycetes which occur in potato tubers. The strands of mycelium to be found around the cells under the scab lesions may be assumed to be of the type which induces the abnormal conditions. But these hyphae seem to be continuous with those surrounding the deeper-placed cells which make up the body of the tuber. One of the striking physiological differences between the hyphae in the cork cambium region and those deeper placed is the formation of a brown stain which spreads from the hyphae of the former to the walls, and even the cytoplasm, of the adjacent cells, but which is lacking in the deeper hyphae or the cell walls which enclose them. The production of this brown stain may not be a permanent physiological character, but it is certainly a very pronounced one. The answer to the question of whether the same species of actinomycetes that are found under the scab spots extend into the tuber would throw much light on the efficacy of seed tuber disinfections; if the true scab types are only near the tuber surface, the disinfectants would destroy or weaken them; if the scab-producing hyphae extend to the interior of the tubers, superficial soaking in a disinfectant would not be any help in controlling the spread of the scab organisms.

Another question suggested but not answered by this work relates to the specific nature of the actinomycetes found in the potato plant, artichoke, beet, etc. Do each of these plants have a set of organisms or a single organism and are the organisms characteristic of the plant in which they are found? When the remains of these plants are destroyed in the soil and become humus, what becomes of the organisms which they have enclosed? Do they adopt a saprophytic type of existence, living on the materials which formerly made up the plants, and lose their pathogenic characteristics? The strains or species of actinomycetes which produce potato scab gradually lose their pathogenicity in soil planted for a number of years to other crops (Lutman, Livingston, and Schmidt, 1936), and fairly clean yields of potato tubers may be harvested from such soil. Since many of the actinomycetes isolated from soil could utilize pectin compounds and therefore must secrete pectinase, the problem would not be one of entrance into the tuber unless the pectin compounds between the potato cells are slightly different from similar compounds between the cells of other plants. The scab-producing organisms, however, show another ability; i.e., the power to stimulate the cork cambium to form the abnormal cork tissue characteristic of scab. The possibility exists that this stimulating character is the one lost by long saprophytic life on decaying plant remains and that some time is required to regain it.

The actinomyces filaments are concentrated in the roots and underground stems of herbaceous plants, probably at least three-fourths of the filaments being located there. The synthesis of proteins in the under-

ground organs of plants has been long known to plant physiologists, this synthesis apparently being one of the functions of roots. Recently, Dawson (1942, 1944) has shown that alkaloids are also synthesized in the roots and later removed to other parts of the plant, especially the seeds. We have absolutely no evidence that the actinomycetes in the roots have anything to do with these syntheses, but since the root and tuber cells are very much like those of other parts of the plant and no glands have ever been discovered, the only striking difference being the abundance of the actinomyces strands between the cells of roots and tubers, it would seem quite plausible that actinomycetes play some part in the manufacture of alkaloids in the underground organs of plants.

The mycorrhizae are not closely related to the actinomycetes, but from their location in the roots together with the same intimate symbiotic relationships which they assume with the cells which they surround, the assumption may be safely made that the functions of the two groups are very similar. By microchemical tests, MacDougal and Dufrenoy (1944) were able to locate auxins and vitamins in and around the mycorrhizal hyphae and found that nucleoproteids are built by them and later transferred for use in building tissues in the roots. The actinomycetes are too small to respond to such tests, but the rich protein content of their mycelium suggests similar functions. The actinomycetes are much wider in their distribution than the mycorrhizae and could perform syntheses in groups of herbaceous plants not provided with mycorrhizae.

It may be rather difficult to ever obtain the answers to these questions, since herbaceous plants are never completely free from actinomyces hyphae and the interplay of host and actinomyces may be so intertwined that a separation of the two would not give results similar to those obtained when they are working symbiotically, as, for instance, the legume nitrogen-fixing organisms will not fix nitrogen in pure cultures. Still, the next logical step would be to isolate actinomycetes from root or tuber tissue, and while some progress has been made by one of the writer's assistants in this direction, the results are still too uncertain to permit detailed statements. Another possible point of attack would be to isolate the organisms which appear in humus, and here it may be said that millions of actinomycetes (apparently all of the *Act. albus* type) can be found in every gram of humus from green potato tops. The objection may be raised that they came from the soil and not from the plant itself.

SUMMARY

1. The writer, using the potato plant especially but also several other plants for comparison, attempts to establish the filamentous nature of the irregular lines which simulate cell walls when sections are viewed under the microscope. They correspond to Mangin's calcium pectate components (middle lamellae) and, with a few exceptions, are identical with them.

2. These filaments branch, seem to push into the cell lumen, and twist and bend in tortuous paths. They may lie one above the other or parallel to each other, or two may cross one another between two walls. They are usually about one micron in diameter, but may become as small as a fraction of a micron.

3. The staining reactions of these filaments indicate a pH of 5.0 to 5.2, while potato tissue has a pH of 5.8 to 6.2. These reactions are in the second range of crystal violet dye, used in Gram's stain, and they result in the filaments staining dark blue while the potato tissue stains reddish violet. The potato scab actinomycetes are known to have a pH of about 5.0 to 5.2.

4. Microchemical tests indicate that these filaments are protein in composition.

5. In view of the known occurrence of actinomycetes in the outer layers of roots and tubers, the morphological resemblance of these filaments to actinomyces strands and their protein nature corroborate the writer's theory that these filaments are actinomyces mycelium.

6. These actinomyces filaments are especially abundant in the outer layers of the tubers and roots of the potato plant, and the stems above ground are almost as completely infected. The tips of young roots and stems contain only a few strands between the cells. Leaves seem to be infected along the phloem of the leaf veins, with only occasional strands occurring between the pulp and palisade parenchyma cells.

7. Potato flower sections are generally infected. The nucellar tissue contains numerous strands, and egg cells are surrounded by strands. Pollen tetrads seem, in some cases, to be held together by them. If fertilization occurs, the resulting young plant would have little chance of escaping infection. These facts would seem to make the infection systemic and hereditary. Young potato plants grown from disinfected seed and in disinfectted soil are found to contain numerous actinomyces filaments.

8. The formation of an abscission layer in the potato flower pedicel is synchronized with the formation of egg mother cells and pollen. All cell walls of the pedicel are infected with actinomyces filaments. The first symptoms of abscission are swellings of the intercellular spaces at the outer margin of a swollen zone on the pedicel. Actinomyces filaments penetrate into these intercellular spaces. Similar swollen places are to be found near vascular bundles, with a similar penetration of filaments. The suggestion is made that an actinomyces may (under the proper stimulus, such as excessive heat) dissolve the intercellular pectins and cause blossom-drop.

9. Walls of regenerated cork cambium cells, on a cut tuber surface, become invaded very quickly by these filaments.

10. Potato scab lesions are associated with strands of actinomyces extending from the abnormal cells of the cork cambium to the interior

of the tuber. The relation of these internal strands to scabbing is not clear, since similar strands have been found in clean tubers grown on land never known to produce scabby tubers. The strands found under the scabs seem to be unusually large and numerous, especially those about five to ten cells below the pathological tissue.

11. The suggestion is made that potato leafroll, which is the foliage stage of net necrosis in the tubers, is the effect of infection by filaments of actinomyces. No artificial inoculations of leafroll have ever been successful, but sucking insects carrying leafroll virus sink their sucking tubes into the phloem of the leaf through the intercellular spaces. The very fine actinomyces filaments in the leaf are located among the phloem cells of the veins. Fragments might be carried on the proboscis of an aphid. No steel or brass instrument could follow the intricate intercellular spaces, as can the pliable, sucking proboscis.

12. The cell walls of Jerusalem artichoke tubers and the enlarged roots of beets, carrots, parsnips, and turnips contain Gram-positive filaments which, while varied in their arrangement, seem quite conclusively to be of the same sort as those occurring in the potato plant.

13. The role of actinomycetes on cell walls is not known, but host plants cannot be indifferent to their intimate and general presence. The suggestion is made that since they are very abundant in the roots of plants, they may take part in the synthesis of alkaloids and proteins.

14. Since large numbers of the soil actinomycetes are pectin-dissolving, the different varieties found in the various host plants may only be modifications of one large species.

15. It would follow from the above that the walls of the higher plants are living, not in the sense that Unger, Sachs, and Ursprung believed them alive, but through the presence and action of strands of an actinomyces or of actinomycetes. The effects of actinomyces filaments surrounding every cell cannot, at this time, be even estimated, but the materials which they withdraw from the cells and the products which they excrete and which must be absorbed by the cells, cannot fail to change the characteristics of the cells.

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